University of Alberta

Purification and characterization of an aminopeptidase and partial purification and characterization of a collagenase from *Mycobacterium tuberculosis*.

by

Laura May Sly ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Medical Microbiology and Immunology

Edmonton, Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Purification And Characterization Of an Aminopeptidase And Partial Purification And Characterization Of a Collagenase From *Mycobacterium tuberculosis* submitted by Laura May Sly in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

We have purified a *Mycobacterium tuberculosis* aminopeptidase to homogeneity. It was purified more than 30,000 fold with a yield of approximately 25 %. It is a monomeric protease of approximately 93 kilodaltons with a pI of 4.1. Protease activity was detected with the synthetic substrate ala-ala-phe-alpha-methylcoumarin. The enzyme was active over a broad pH range, 5.5 - 8.5, with optimal activity at pH 7.0 and stable over a temperature range of 20°C - 48°C, with optimal activity at 32°C. *M. tuberculosis* aminopeptidase activity was inhibited by aminopeptidase inhibitors, amastatin and bestatin, metalloenzyme inhibitors EDTA and 1,10-phenanthroline, and the zinc metalloprotease inhibitor, Zincov™. It was not inhibited by serine, cysteine or aspartic protease inhibitors. Enzymatic activity was dependent on the presence of metal cations. Optimal activity occurred with concentrations of $ZnCl_2$ in excess of 50 μM. The enzyme hydrolyzed a wide range of α-methylcoumarin (AMC) substituted amino acids with highest activity against arg-AMC and high activity against substrates with basic amino acid side chains at the P1 position. Km values for R-AMC, L-AMC and P-AMC were 4.7, 19.2 and 36.2 μM, respectively. Vmax values were 317, 258 and 9.9 μmoles min^{-1} mg^{-1}, respectively. Turnover rates were 491, 480 and 15 s^{-1}, respectively. The N-terminal sequence of the purified protein was similar to the translated N-terminal sequence of a potential aminopeptidase from *M. tuberculosis* strain H37Rv. Two differences noted were the absence of a predicted N-terminal met and the substitution from leu to asp at position 2 in the H37Ra sequence.

We have partially purified a *Mycobacterium tuberculosis* collagenase. It was purified more than 1,000 fold with a yield of approximately 40 %. The molecular weight of the non-denatured protease is 53 kilodaltons with a pI of 3.4. Protease activity was detected with the synthetic substrate N-succinyl-gly-pro-leu-gly-pro-

alpha-methylcoumarin. The enzyme was active over a broad pH range, 6.0 – 10.0, with optimal activity at pH 8.5 and stable over a temperature range of 20°C - 48°C, with optimal activity at 34 – 37°C. *M. tuberculosis* collagenase activity was inhibited by serine protease inhibitors 3,4-DCI and PMSF but not by TLCK or TPCK. It was not inhibited by metallo-, cysteine or aspartic protease inhibitors.

Dedication

I dedicate this thesis to my partner, Robert Cecil Sobotkiewicz, and our daughter, Isabel Sly Sobotkiewicz. They have always been a tremendous source of support and inspiration.

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I would like to thank my supervisor, Dr. Jim Talbot, for the tremendous experience I've had in his laboratory. He is the first person in my life that I would call mentor, and I thank him for that.

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List of Abbreviations

A280	absorbance at 280 nanometers
A553	absorbance at 553 nanometers
A600	absorbance at 600 nanometers
AAF-AMC	alanine-alanine-alanine-alpha-methylcoumarin
AEC	anion exchange chromatography
amastatin	[(2S,3R)-3-amino-2-hydroxy-5-methyl-hexanoyl]-val-val-asp-OH
AMC	alpha-methylcoumarin

Amino acids:

three-letter code	one-letter code	Amino acid
ala	A	Alanine
arg	R	Arginine
asn	N	Asparagine
asp	D	Aspartic acid
cys	C	Cysteine
gln	Q	Glutamine
glu	E	Glutamic acid
gly	G	Glycine
his	H	Histidine
ile	I	Isoleucine
leu	L	Leucine
lys	K	Lysine
met	M	Methionine
phe	F	Phenylalanine
pro	P	Proline
ser	S	Serine
thr	T	Threonine
trp	W	Tryptophan
tyr	Y	Tyrosine
val	V	Valine

bestatin	[(2S,3R)-3-amino-2-hydroxy-4-phenyl-butanoyl]-leu-OH

Da	dalton
DMSO	dimethyl sulfoxide
3,4-DCI	3,4-dichloroisocoumarin
E-64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane,N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]-agmatine
ECF	extracellular filtrate concentrate
EDTA	ethylenediaminetetraacetic acid
ET	electrophoretic type
FPLC	fast performance liquid chromatography
FU	fluorescence units
HIC	hydrophobic interaction chromatography
hr	hour
IEC	ion exchange chromatography
IgG	immunoglobulin G
kDa	kilodalton
Km	Michaelis-Menten constant
LDM	Laemmli's digestion mix
leupeptin	N-acetyl-leu-leu-arg-al
MDR-TB	multi-drug resistant tuberculosis
min	minute
mL	milliliter
MLEE	multilocus enzyme electrophoresis
Mr	molecular weight

NaCl	sodium chloride
N-succ-GPLGP-AMC	N-succinyl-glycine-proline-leucine-glycine-proline-alpha-methylcoumarin
P1 position	the amino acid on the N-terminal side of a peptide bond that is cleaved by a protease
P1' position	the amino acid on the C-terminal side of a peptide bond that is cleaved by a protease
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pepstatin	isovaleryl-val-val-AHMHA-ala-AHMHA (AHMHA = (3S,4S) 4amino-3-hydroxy-6-methyl-heptanoic acid]
Phenyl HI SUB	phenyl sepharose hydrophobic interaction chromatography column, with .34 mmoles phenyl per mL swollen gel
Phenyl LO SUB	phenyl sepharose hydrophobic interaction chromatography column with .12 mmoles phenyl per mL swollen gel
PMSF	phenylmethylsulfonyl fluoride
Q Seph FF	Q Sepharose Fast Flow anion exchange chromatography column
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
son	sonicate
Superdex 200	Superdex 200 size exclusion chromatography column
TB	tuberculosis
TLCK	L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone HCl
TPCK	L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone
Vmax	the maximum rate of reaction of an enzyme

w/v	weight per volume
Zincov™	[2-(N-Hydroxycarboxamido)-4-methylpentanoyl-L-Ala-Gly amide]

Chapter I. Introduction

I.1. *Mycobacterium tuberculosis*

I.1.a. The organism

Mycobacterium tuberculosis is a straight or slightly curved bacillus, 0.2 – 0.7 μm wide and 1 – 10 μm long. It is non-motile and does not produce capsule or spores. It stains faintly Gram-positive but is more readily stained by the Ziehl-Neelsen method with carbol fuschin. It is resistant to destaining with acid and alcohol and so is called acid and alcohol fast (Grosset, 1993; Holt *et al.*, 1994). Its cell wall is approximately 20 nm thick and it has no outer membrane and thus, is more closely related to the Gram-positive bacteria. Its cell wall is unusual in that it is exceptionally thick and has a high lipid content (Grosset, 1993).

M. tuberculosis growth is extremely slow, requiring 2 to 4 weeks for colonies to appear on solid media and 10 to 14 days for detection of growth in liquid media by release of $^{14}CO_2$ from labeled growth substrates. It is a strict aerobe. Preferred carbon sources are glycerol, pyruvate and glucose, in that order. It requires iron for growth, which is usually provided in the form of ferric ammonium citrate *in vitro*. It can use nitrogen from several small molecules including asparagine, glutamine, aspartic acid, glutamic acid, alanine and ammonium salts. It also requires trace amounts of potassium, magnesium, sulfur, phosphorous, zinc and manganese (Grosset, 1993).

The genome of the virulent strain H37Rv is 4,411,529 base pairs and contains 3924 open reading frames. It is the second largest bacterial genome sequenced, next to *Escherichia coli*. It has an exceptionally high guanosine + cytosine content, 65.6 % (Cole *et al.*, 1998). Functions for approximately 40 % of the genes are predicted from

strong DNA sequence homology to known genes, 44 % of the genes are suggested from lower levels of sequence homology to known genes and 16 % are of unknown function (Cole *et al.*, 1998).

I.1.b. The disease

Mycobacterium tuberculosis is primarily a respiratory pathogen and its primary route of transmission is as an airborne aerosol. Active disease is accompanied by cough, fever, weight loss and night sweats. After infection, an immunocompetent individual has approximately 10 % chance of developing disease in their lifetime, but susceptibility to disease is influenced by host factors. Host factors, which are important, include age, immune status and coinfection with other diseases. In the immunocompetent host, 85 % of disease is pulmonary and only 15 % of disease is associated with extrapulmonary sites (Hopewell, 1994; Hopewell, 1995).

I.1.c. Epidemiology

Tuberculosis is a global health threat. There are an estimated 2 billion people infected world wide, more than 1/3 of the world's population (Williams and Douglas-Jones, 1995). It is the leading cause of death among bacterial infectious agents world-wide (Williams and Douglas-Jones, 1995) resulting in 8 to 10 million new cases and 2 to 3 million deaths per year (Huebner and Castro, 1995; Shinnick *et al.*, 1995). There was a steady decline in the number of new cases reported annually in the United States until 1985 (Bass, 1995). In 1993, there was a decrease in the number of cases due to vigilant observed therapy programs but it was estimated between 52,000 and 64,000 cases occurred above what was expected had the consistent decline continued (Bass, 1995; Huebner and Castro, 1995).

I.1.d. Tuberculosis in Canada and in Alberta

In Canada from 1980-1988, there was a decrease in incidence of disease. Since that time, the number of cases of disease has been steady (Fig. I-1). Incidence of disease has continued to decline in both non-aboriginal and aboriginal Canadian-born populations. The plateau observed in the total number of cases is due to an increase in incidence of disease in foreign-born Canadians (Health Canada, 1995).

In Alberta from 1969 to 1989, there was a decrease in incidence of new disease. That trend has stopped and the incidence of disease has been erratic since that time (Fig. I-2). The incidence rate is higher in foreign-born as well as aboriginal Albertans. From 1986 to 1995, case numbers per 100,000 population range from 1.2-3.3 for non-aboriginal, Canadian-born Albertans; 16.5 - 29.3 for foreign-born Albertans and 31 - 152 for aboriginal Albertans (Alberta Health, 1995). Although Canada and Alberta have not seen a large increase in incidence of disease, the decrease in incidence of disease has stopped, a trend which has been observed world wide.

I.1.e. Tuberculosis and human immunodeficiency virus (HIV)

The world epidemic of HIV has increased the importance of TB as a health care issue. In 1990, an estimated 3 million people were coinfected with both agents (Garcia *et al.*, 1995). Coinfection is seen not only geographically and in individual patients, but in the same macrophages as well (Williams and Douglas-Jones, 1995). HIV patients with pulmonary TB may be difficult to diagnose, as the symptoms of the two diseases are similar (Huebner and Castro, 1995). Further complicating diagnosis, HIV leads to anergy in the PPD skin test in approximately 50 % of the HIV positive population (Williams and Douglas-Jones, 1995; Garcia *et al.*, 1995). Sputa are collected from

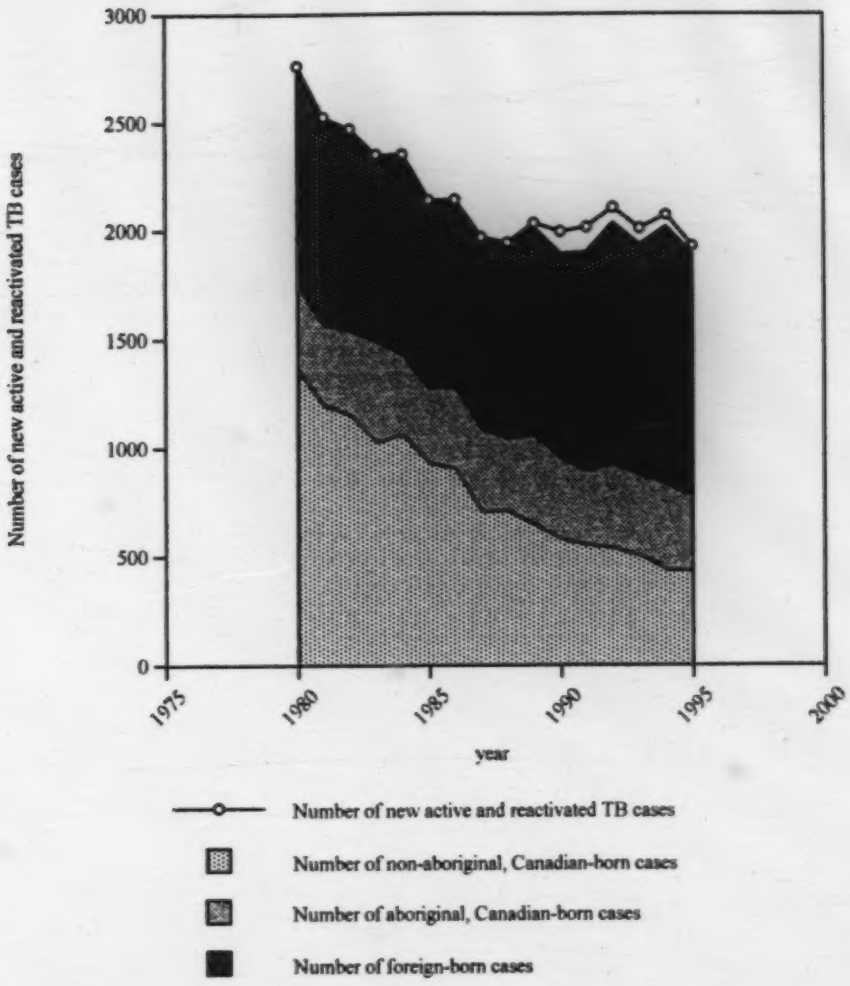

FIG. I-1. The number of new active and reactivated TB cases in Canada from 1980-1995. Cases are broken down into non-aboriginal and Canadian-born patients, aboriginal and Canadian-born patients and foreign-born patients. Unfilled areas under the graph represent individuals where birth place was unknown. Data is from the Tuberculosis in Canada 1995 Annual Report, Health Canada, Health Protection Branch, Laboratory Centre for Disease Control.

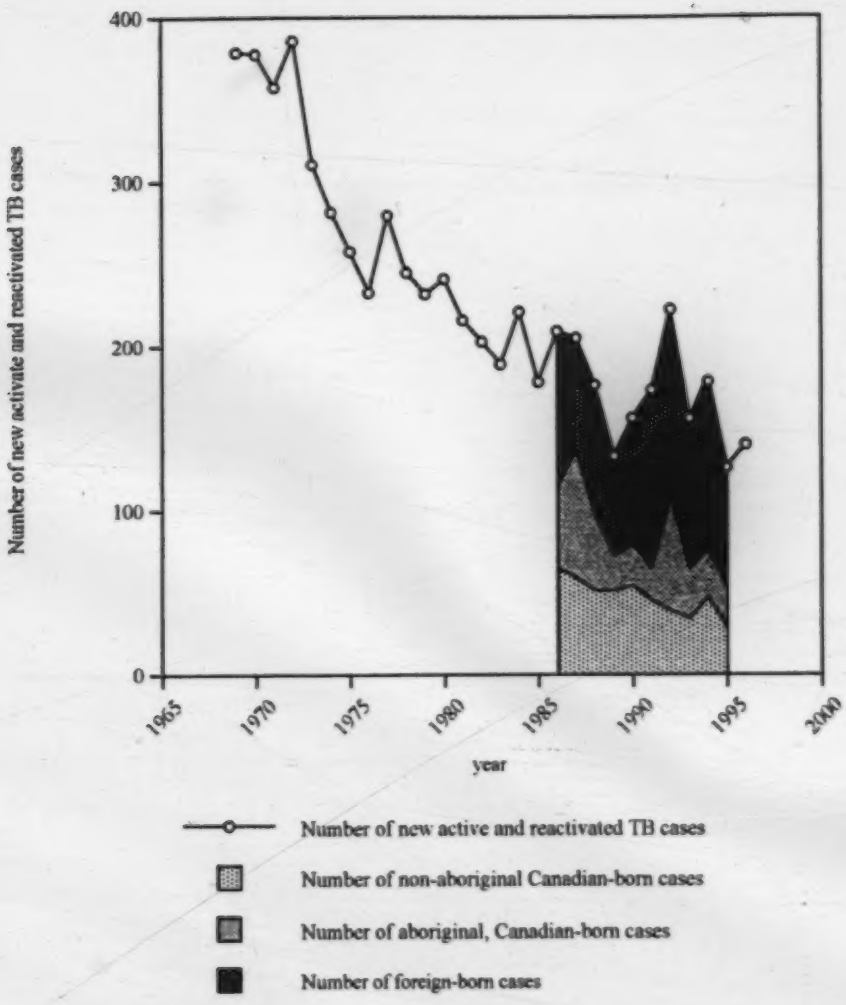

FIG. I-2. The number of new active and reactivated TB cases in Alberta from 1969 to 1996. Cases from 1986 to 1995 are broken down into non-aboriginal and Canadian-born patients, aboriginal and Canadian-born patients and foreign-born patients. Data is from the Alberta Health Care TB services annual report, 1995. Data from 1996 is a personal communication from Joanne Seglie, Alberta Health, TB services.

patients for diagnosis by culture. Radiographs of lungs are useful in identifying tuberculous lesions but both of these may be negative in as many as 12 % of HIV infected individuals.

The increased health threat of coinfection is partially due to an increase in incidence of tuberculous infection in the HIV positive population. Non-HIV infected people who are PPD positive have a 10 % chance of developing active TB during their lifetime (Hopewell, 1995) while people with HIV have an 8 - 10 % chance of developing active TB annually (Bass, 1995). 50 % of the HIV positive population will develop active TB during their lifetime (Garcia *et al.*, 1995). The result is an additional 300 thousand cases of active TB per annum and 120 - 150 thousand deaths annually (Garcia *et al.*, 1995).

There are two additional consequences of coinfections with HIV and TB. First, TB occurs as disseminated disease in the HIV population more frequently. Extrapulmonary disease is seen in only 15 % of non-HIV infected individuals (Hopewell, 1995) but occurs in 62 % of the HIV-positive population, with or without pulmonary involvement (Hopewell, 1995; Williams and Douglas-Jones, 1995). Secondly, TB can have devastating consequences for HIV-positive patients particularly early in HIV disease as TB-activated host cytokines essential to the antimycobacterial immune response have been shown to increase HIV replication in monocytes and lymphocytes *in vitro* (Wallis *et al.*, 1992; Williams and Douglas-Jones, 1995).

I.1.f. Multi-drug resistant tuberculosis (MDR-TB)

The resurgence of TB has been concomitant with the emergence of multi-drug resistant strains (Huebner and Castro, 1995). Inappropriate and ineffective use of

antimycobacterial chemotherapeutics over the past decades has led to MDR-TB and recent resurgence of disease has increased the visibility of the problem (Ellner, 1995). Drug resistance arises from spontaneous mutations which occur once in every 10^5 to 10^8 bacterial cells. Inappropriate anti-tuberculosis chemotherapy including use of the wrong antibiotic, too few effective antibiotics in the combination, as well as non-compliance from patients with appropriate chemotherapy contribute to the selection of resistant organisms, allowing their proliferation in the host and transfer to other susceptible individuals (Huebner and Castro, 1995).

MDR-TB is resistant to both isoniazid and rifampin, drugs used as first-line antibiotics when infection is identified. MDR-TB has also been identified which is resistant to ethambutol and streptomycin, two additional first-line antibiotics, as well as ethionamide, kanamycin, and rifambutin (Huebner and Castro, 1995). The majority of MDR-TBs have been identified in, but are not limited to, the HIV-positive population (Ellner, 1995). More than 300 MDR-TB cases have been reported during 8 outbreaks in the US and mortality rates ranged from 43 to 93 % with the time from diagnosis to death being 4 to 16 weeks (Huebner and Castro, 1995). In the non-HIV positive population, there is only a 50 % cure rate while in the HIV-infected population, the disease is incurable (Ellner, 1995). MDR-TB has also led to the occurrence of nosocomial outbreaks, particularly in the HIV-infected population (Ellner, 1995).

I.2. Mycobacterial virulence

Mycobacterium tuberculosis is not known to produce toxins. Its virulence is dependent on its ability to survive and multiply within the human host (Grosset, 1993). Determinants of cell entry, survival and latency are therefore critical pathogenic

determinants in *M. tuberculosis* (Schlesinger, 1996). Further, mycobacterial interactions with host cells are critical to the pathogenesis of the organism (Hasan *et al.*, 1997).

I.2.a. The mycobacterial phagosome

When inert particles enter the phagosome, the phagosome matures, a process that includes acidification of its interior to a pH of approximately 6.0. The phagosome fuses with a lysosome and acidification to pH 5.5 continues. The normal result of phagosome-lysosome fusion is the killing and degradation of an internalized bacterium (Clemens, 1996). However, in the case of *M. tuberculosis* and *M. avium*, phagosomes are only mildly acidified to a pH of 6.0 - 6.5 and the phagosome does not fuse with lysosomal particles, preventing continued acidification of the phagolysosome and subsequent lysosomal-dependent killing. There are four factors proposed to contribute to the arrest of the mycobacterial phagosome maturation.

I.2.b. The type of host cell

Upon initial infection with *M. tuberculosis* the first line of attack is resident, differentiated, alveolar macrophages (Schlesinger, 1996). These macrophages have high respiration, altered uptake mechanisms for foreign organisms and surface immunoglobulin. During later stages of infection, the primary defense is circulatory derived monocytes that may or may not have differentiated before encountering the microorganism. Differentiation depends on time and exposure to local conditions in the lung (Schlesinger, 1996). Pancholi *et al.* (1993) showed that CD4+ T-cells have antimicrobial activity against TB contained within macrophages but monocytes are sequestered from CD4+ T-cells. This suggests a mechanism by which the type of host

cell infected may allow *M. tuberculosis* to persist within the human host (Pancholi *et al.*, 1993).

I.2.c. Mechanism of internalization

M. tuberculosis uptake occurs by receptor-mediated phagocytosis which can involve any of four different classes of host receptors; the Fc receptor, complement receptors 3 and 4, and the mannose receptor (Schlesinger, 1996). The avirulent strain, H37Ra, which is unable to persist in the phagosome, is not taken up by the mannose receptor-mediated pathway like virulent strains H37Rv and Erdmann, suggesting that route of entry may influence the intracellular fate of the organism.

An invasion factor has been described in *M. tuberculosis* and localized to a single gene fragment (Arruda *et al.*, 1993). It is hypothesized to be similar to the *Listeria* internalin and *Yersinia* invasin proteins, both bacterial surface associated proteins which bind specific host cell structures (Pizarro-Cerda *et al.*, 1997).

I.2.d. Prevention of acidification of the phagosome

Two mechanisms by which the interior of *M. tuberculosis* infected phagosomes are prevented from acidification have been proposed. Normally, acidification occurs because the phagosome loses the Na^+K^+ATPase, which would prevent acidification. Fusion with lysosomal particles, containing a vacuolar proton pump, enhances acidification. The first mechanism is inhibition of one or both of these maturation processes (Sturgill-Koszycki *et al.*, 1994). This suggests a bacterial-mediated change in phagosome maturation preventing acidification of the phagosome.

The second hypothetical mechanism is the antagonism of acidification of the phagosome by the production of ammonia by the bacterium. Enzymes from

M. tuberculosis produce sufficient ammonia to block phagosome-lysosome fusion (Gordon *et al.*, 1980). Similar amounts of ammonia inhibited phagosome-lysosome fusion in phagosomes containing yeast cells, and, fusion was restored when ammonia was removed (Gordon *et al.*, 1980). *M. tuberculosis* ammonia-producing enzymes with substrates present in the phagosome include arginase, asparaginase, glutaminase and urease. Arginase digests arginine with the addition of water to ornithine and urea, which must then be broken down by urease to ammonia and carbon dioxide. Asparaginase and glutaminase catabolize asparagine and glutamine to aspartate and glutamate, respectively, directly releasing ammonia (Gordon *et al.*, 1980).

I.2.e. Mycobacterial and host cell protein regulation

Studies of *M. avium* invasion into macrophage cell lines and epithelial cells suggest that selected proteins of the organism and host are upregulated after internalization occurs and that the proteins produced differ according to host cell type and time after internalization (Bermudez and Petrofsky, 1997). Increased amounts of four *M. tuberculosis* proteins, with molecular weights of 93, 65, 55 and 33 kDa, were seen two hours after internalization by macrophages. After 4 hours, 6 more proteins ranging in molecular weight from 31 - 70 kDa were also seen. In epithelial cells, upregulation of four *M. tuberculosis* proteins with molecular weights ranging from 25 - 72 kDa was seen and this pattern did not change with time (Bermudez and Petrofsky, 1997). There were also changes observed in host macrophage proteins expressed after internalization of *M. bovis* BCG suggesting that the macrophage undergoes regulatory changes after it is invaded (Hasan *et al.*, 1997).

I.3. Proteases

Proteases are enzymes which hydrolyze peptide bonds in peptides or proteins. There are four classification schemes which describe proteases. The first classifies them according to their active site residue as serine, cysteine, aspartic or metalloproteases. This classification can be deciphered experimentally by inhibitors effective against the protease and provides insight into their mechanism of action. Secondly, they are described on the basis of their pH optima as neutral, acidic or basic proteases. Thirdly, they are described by whether they begin cleavage at they end of a protein (exopeptidases), which may cleave from the amino-terminus (aminopeptidases) or the carboxyl-terminus (carboxypeptidases), or internally (endopeptidases). Fourthly, they are described by the substrates that they cleave. For example, an elastase can cleave elastin and a collagenase can cleave collagen.

I.3.a. Bacterial aminopeptidases

Bacterial aminopeptidases may have broad or narrow specificity. They can be located in the cytoplasm, cell membrane, periplasm or secreted. All bacterial aminopeptidases exhibit Michaelis-Menten kinetics with the exception of Aminopeptidase A from *Lactococcus lactis*, which is an allosterically activated hexamer with identical subunits. Bacterial aminopeptidases are most commonly metalloproteases, though they can also be cysteine and serine proteases (Gonzales and Robert-Baudouy, 1993). These proteases may play a role as housekeeping enzymes providing the final step in catabolism of exogenously or endogenously supplied peptides or proteins. Specific functions assigned to some bacterial aminopeptidases include cleavage of N-terminal methionine from proteins during processing and cleavage of N-terminal

pyroglutamic acid (Ben-Bassat *et al.*, 1987; Szewezuk and Mulczyk, 1969). The Pep N protease from *E. coli* has been implicated in stabilization of the multicopy ColE1 plasmid in *E. coli* although it may only have a structural role (Gonzales and Robert-Baudouy, 1993). Finally, bacterial aminopeptidases may play a role in degradation of toxic peptides or proteins or physiologically important peptides or proteins (Gonzales and Robert-Baudouy, 1993).

I.3.b. Bacterial proteases as virulence factors

Goguen *et al.* (1995) separate the potential roles of proteolysis in enhancing virulence of a pathogen into three categories. Proteolysis due to enzymes with broad specificity directed against host proteins that are abundant was termed high level proteolysis. This type of proteolysis would provide the organism with peptides or amino acids to use in metabolism or directly for biosynthesis while destroying larger amounts of host tissue (Goguen *et al.*, 1995) and important host proteins (Maeda, 1996). Proteins destroyed could include host immune proteins moving into the site of infection thus, in a non-specific process, aiding the bacterium to avoid the host immune response (Goguen *et al.*, 1995; Maeda, 1996). The *Pseudomonas aeruginosa* elastase is a broad specificity, secreted protease. It has been implicated in corneal damage during keratitis in the rabbit model and lung injury in hamsters from experiments performed with purified protease (Goguen *et al.*, 1995). Its role in these disease processes has not been proven using defined mutants for this protease.

Two additional roles outlined by Goguen *et al.* (1995) are the inhibition of the host immune response or, in contrast, the activation of an inappropriate response. In the first case, this may be accomplished by inactivating regulatory proteins key in the host

immune response resulting in an ineffective response and allowing the spread of the organism. The plasmid-encoded, outer membrane P1a protease of *Yersinia pestis* may function in this way. P1a$^-$ mutants produced local, self-resolving lesions after subcutaneous injection into a mouse while wild type organisms produced overwhelming, systemic infection. In addition, mutant lesions contained large numbers of host inflammatory cells which are absent from wild type lesions. Although the specific target(s) for the P1a protease remains to be elucidated, the protease interferes with host inflammatory response, leading to dissemination of the organism (Goguen *et al.*, 1995). The activation of an inappropriate immune response may be accomplished by activating regulatory proteins resulting in interference with the host immune response and allowing the spread of the organism (Goguen *et al.*, 1995). Exotoxin B, from *Streptococcus pyogenes*, may function in this manner. Exotoxin B is the zymogen or inactive precursor form of a cysteine protease that can cleave pre-IL-1β to produce active IL-1β, an inflammatory mediator. This could contribute to local inflammation and systemic shock, both of which have been observed during *S. pyogenes* infection. The evidence which supports a role for this cysteine protease in disease is that cysteine protease inhibitors protected mice from fatal infection following intra-peritoneal injection (Goguen *et al.*, 1995).

I.4. Proteases of Mycobacterium species

I.4.a. Evidence for the presence of proteases in *Mycobacterium tuberculosis*

Reich *et al.* (1981) reported the recycling of *M. tuberculosis* culture filtrate proteins. Using ^{14}C-labeled excreted tuberculoproteins, they showed that proteolytic activity present in the culture supernatant allowed the cells to incorporate radiolabeled

amino acids into intracellular proteins. Further, after washing these cells, the radioactivity was shown to reappear in the culture supernatant. This may have been due to cell lysis and/or excretion of labeled proteins. The recycling of culture filtrate tuberculoproteins (Reich *et al.*, 1981) indicates the presence of proteases and provides a mechanism by which the bacterium may use proteases to provide nutritive requirements.

Rowland *et al.* (1997) identified an elastolytic and caseinolytic activity present in *M. tuberculosis* four week old culture filtrates. Elastin is a large fibrous protein important for lung elasticity and its destruction is associated with susceptibility to infection and chronic lung disease (Rowland *et al.*, 1997). Elastolytic and caseinolytic activities were observed concomitantly during culture growth. They had a pH optimum between 6.5 and 7.5 and a complex inhibition profile. Activities were inhibited by EDTA, EGTA, DTT, 1,10-phenanthroline, Zincov™ and TPCK. Activities were also partially inhibited by PMSF, TLCK and E-64 (Rowland *et al.*, 1997). From this inhibition profile, Rowland *et al.* (1997) concluded that the proteolytic activity was due to a Ca^{2+}-dependent elastolytic metalloprotease. The activity described could also be due to the presence of more than one protease class resulting in partial inhibition by serine and cysteine type protease inhibitors.

I.4.b. The *Mycobacterium tuberculosis* aminopeptidase

An *M. tuberculosis* aminopeptidase has been previously identified (Gleisner and Ramthun, 1981). It was found in whole cell lysates of *M. tuberculosis* strains H37Rv, H37Ra and a clinical isolate. A summary of its characteristics is given in Table IV-4.

Multilocus enzyme electrophoresis (MLEE) has been used to characterize *Mycobacterium avium* Complex (Wasem *et al.*, 1991; Yakrus *et al.*, 1992). MLEE has

been used to estimate the genetic diversity in several biologically important species including *E. coli*, *Salmonella newport*, *Bordetella* spp., *Haemophilus influenza*, *Neisseria meningitidis*, *Legionella* spp., *Pseudomonas aeruginosa*, *Streptococcus* spp., *Yersinia ruckeri*, *Klebsiella oxytoca*, and *Rhizobium* spp. (Selander *et al.*, 1986). Whole cell lysates of bacteria are separated on starch gels, which are then sliced into 1-2 mm thick lengths and each slice is used to assay for a cellular enzyme. Polymorphism of these water-soluble enzymes is used to evaluate relatedness between species and strains that are classified into electrophoretic types (ETs).

Wasem *et al.* (1991) screened 23 MAC strains, which include species *avium* and *intracellulare* as well as 10 other *Mycobacterium* spp., including *M. tuberculosis*, for 20 enzymes. Among the enzymes evaluated was leucine aminopeptidase (LAP) which is capable of cleaving an N-terminal leucine from the substrate, L-Leucine-β-naphthylamide hydrochloride. One isoform of this enzyme was present in *M. tuberculosis* and it may be the same enzyme described by Gleisner and Ramthun (1981). Up to 5 isoforms of this enzyme were found in all other mycobacterial isolates that were tested (Wasem *et al.*, 1991).

MAC isolates evaluated had between 2 and 4 isoforms, *M. paratuberculosis* had 2, *M. scrofulaceum* had 3 or 5, *M. kansasii* had 1, *M. chelonae* had 5, *M. gordonae* had 1, *M. xenopi* had 4, *M. smegmatis* had 5 and *M. fortuitum* also had 5 isoforms of LAP (Wasem *et al.*, 1991). A similar study was performed by Yakrus *et al.* (1992) to characterize *M. avium* isolates of serotypes 4 and 8 isolated from patients with AIDS. In this study, isolates with 0, 1 and 2 isoforms of LAP were identified (Yakrus *et al.*, 1992).

I.4.c. The *Mycobacterium tuberculosis* collagenase

A second proteolytic activity in *M. tuberculosis* is a collagenolytic enzyme, which was first identified in 1957. Takahashi (1957) described the isolation of this enzyme to homogeneity as assessed by amido black staining of a polyacrylamide tube gel. The enzyme was isolated from *M. tuberculosis* culture supernatants by three steps: i) precipitation of the crude filtrates by 40% w/v ammonium sulfate with 4 mM $CaCl_2$, ii) starch zone electrophoresis at pH 8.5 in Veronal acetate buffer with 4 mM $CaCl_2$, which removed the casein hydrolyzing activity, and iii) DEAE-cellulose chromatography eluting with an increasing step-wise salt gradient. The purified enzyme was characterized and found to degrade both collagen from calf skin, azocoll, and a synthetic substrate for collagenase, Z-G-P-L-G-P. The molecular weight of the purified product was found to be 77 kDa by sedimentation equilibrium and the pH optimum of the activity was between 6 and 8 (Takahashi, 1957).

I.4.d. Other mycobacterial proteases

A potential serine protease gene was sequenced from *M. avium* subsp. *paratuberculosis* (Cameron *et al.*, 1994). The gene was cloned after screening an expression library with sheep serum from a sheep previously infected with *M. avium* subsp. *paratuberculosis*. The sequence encoded a 34 kDa protein which had a motif identical to that flanking the active serine residue number 195 of trypsin. It showed 30 % homology to the *E. coli*, *Salmonella typhimurium*, *Brucella abortus* and *Rochalimaea henselae* HtrA proteins, also predicted serine proteases (Cameron *et al.*, 1994).

Finally, there is an intracellular dipeptidase that has been purified from *M. phlei*

(Plancot and Han, 1972). It had highest affinity for L-leu-gly substrate, required metal cations for activity, had a pH optimum of 9.5, and a molecular weight of 88 kDa by gel filtration and 45 kDa by SDS-PAGE (Plancot and Han, 1972).

I.4.e. Proteases predicted from the genome sequence *of Mycobacterium tuberculosis* H37Rv

There are 29 genes found in *M. tuberculosis* H37Rv, which are predicted to code for proteases or protease subunits by their amino acid sequence homology to known protease sequences (Cole *et al.*, 1998). There are also many open reading frames (628), for which functions were not predicted from sequence homologies. Therefore, the list of predicted protease genes provided by Cole *et al.* (1998) might not be complete. Further, it remains to be elucidated whether predicted genes produce proteins and whether the proteins function as predicted.

I.5. Protein purification

Protein purification can be divided into three stages. The stages are enzyme capture, intermediate purification and final purification (Kaleja, 1997). It is important that a rapid, sensitive and specific assay is available for the detection of the protein of interest during purification. Speed is particularly important in the absence of protease inhibitors to prevent degradation of the target protein during purification (Linn, 1990).

It is critical to establish goals for the isolation procedure including the amount of protein required, the degree of purity and whether it must be biologically active. Harsh conditions are imposed in some very effective isolation procedures and may result in a loss of biological activity (Linn, 1990). Alternatively, one can perform the purification and then establish whether activity is recoverable afterwards. Clear goals for

purification allow chromatography conditions to be selected, with acceptable balance between sacrificing capacity (amount of protein required) for resolution (degree of purification achieved in each procedure; Linn, 1990; Roe, 1990; Kaleja, 1997).

To purify a target protein from a complex mixture, separation on the basis of a combination of different properties of the protein of interest may be required. Properties of the protein which can be used include its charge, hydrophobicity, size and affinity for other molecules. Each of these properties may be exploited by a variety of separation techniques. For example, the charge on a protein can be used to purify it by ion exchange chromatography, cation of anion, electrophoresis or chromatofocusing. Therefore, surveying different separation techniques based on a single property may be a profitable initial exercise. All of the properties of the protein can be exploited for the native protein and the denatured protein. A flow scheme for purification of proteins is shown in Appendix 2, FIG. A2-1.

I.5.a. Ion exchange chromatography (IEC)

Ion exchange chromatography separates proteins on the basis of exposed charge. It can be run in the absence or presence of non-ionic denaturants. The column matrix has charged groups which interact directly with oppositely charged groups on the native (surface exposed) or denatured protein. Elution from the column matrix is performed by decreasing the ionic interactions between the protein and the matrix. This can be accomplished by increasing the conductivity of the eluate or changing the pH of the eluting buffer thus decreasing the charge on the protein or column matrix (Roe, 1990). Ion exchange is a powerful technique with high protein capacity often used early in intermediate purification (Linn, 1990).

There are two types of ion exchange chromatography, anion exchange and cation exchange. Both rely on interaction between charged groups on the column matrix and protein. Positive charges on proteins are conferred by the amino acids his, lys, arg and, to a lesser extent, the N-terminal amino group. These charges contribute to protein interaction with anion exchangers. Negative charges on proteins are conferred by amino acids asp, glu and, to a lesser extent, cys and the C-terminal carboxylic acid group. Negative charges contribute to interaction with cation exchangers (Roe, 1990).

Ion exchangers can be further subdivided into strong and weak categories. These categories do not reflect binding ability at neutral pH, but rather, describe the stability of ionization of the column matrix sidegroups over a wide pH range. Strong ion exchangers are completely ionized over a wide pH range while weak ion exchangers are not. The degree of dissociation of weak ion exchangers and hence ion exchange capacity, varies with pH (Roe, 1990).

The pH at which ion exchange is carried out must be selected. To promote strong binding between the column matrix and the protein of interest, it is recommended that a pH of 1 unit greater than the pI of the protein is used in anion exchange chromatography, or 1 unit less than the pI of the protein is used in cation exchange chromatography (Roe, 1990). However, the ability of a protein to bind to the column matrix must be determined experimentally. Proteins with a pI close to 7 may benefit from purification by anion or cation exchange columns and the choice may need to be determined experimentally.

I.5.b. Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography separates proteins on the basis of their

surface hydrophobicity. The column matrix has hydrophobic groups which can interact with hydrophobic amino acid side chains like those possessed by amino acids trp, phe, ile, leu, tyr, pro, met, val and ala. Hydrophobic amino acid side chains on protein surfaces are predicted to occur in patches (Roe, 1990). Binding of a protein to a hydrophobic matrix is entropy driven. The release of bound surface water from the protein increases the free energy of the reaction leading to spontaneous binding to the column matrix (Roe, 1990). Proteins are eluted from the matrix by decreasing the salt concentration of the column buffer (Roe, 1990). HIC columns have an intermediate capacity and are often used during intermediate purification (Linn, 1990).

Different columns are available with different hydrophobic groups bound to Sepharose. The optimal degree of hydrophobicity of the matrix must be determined experimentally by screening the matrices (Kaleja, 1997). Optimal column running conditions for separation must also be determined experimentally (Kaleja, 1997). Temperature and pH affect protein binding to the matrix. At lower temperatures, hydrophobic interactions are decreased and at a pH approaching the pI of the protein, where there would be no net charge on the protein, the hydrophobic interactions are increased (Roe, 1990).

I.5.c. Size exclusion chromatography (SEC)

Size exclusion chromatography separates proteins on the basis of their molecular size. This technique can be performed in the presence of denaturants which separates multimers into their smaller molecular weight subunits and may eliminate the influence of shape of the native protein on elution (Prenta, 1990). SEC uses a solid phase support containing pores of different molecular sizes that sieve the proteins as they pass through

the column. The theoretical maximum elution volume, after which all material from the column is eluted, is therefore one column volume. Very small molecules have access to all matrix pores so spend the largest amount of time in the column while large molecules are excluded from some of the matrix pores and are eluted earlier (Prenta, 1990). Resolution from the column is proportional to the square root of the column volume and elution time is proportional to column volume (Kaleja, 1997). Interaction with the column matrix can be minimized for optimal separation by including salt in the running buffer (Kaleja, 1997). SEC is an intermediate capacity column and is often used during intermediate or final purification steps (Linn, 1990).

I.6. Objectives of this study

In this study, we attempted to identify excreted proteases from *M. tuberculosis*. Excreted proteases could act as virulence factors by cleaving host proteins thereby contributing to tissue damage during infection, and may be important in the host immune response to tuberculosis. Potentially excreted proteases were identified by the presence of high activity against two synthetic peptide substrates in crude 7 day old culture filtrates. We attempted to purify the proteins responsible for these activities from extracellular filtrate concentrate and to characterize them to gain a better understanding of their potential role in *M. tuberculosis* growth and pathogenesis.

In this study, we describe the purification and characterization of an aminopeptidase from *M. tuberculosis* including its optimal activity, substrate specificity and N-terminal sequence. We also describe the partial purification of an *M. tuberculosis* collagenase and characterization of the crude and partially purified activity.

Chapter II. Materials and Methods

II.1. Bacterial strains

Mycobacterium tuberculosis strain H37Ra (ATCC 25177), an avirulent strain, was used as the source of proteases.

II.2. Buffers, solutions and media

Recipes for buffers, solutions and media used in the production, purification and characterization of the *M. tuberculosis* aminopeptidase and collagenase are provided in Appendix 3.

II.3. Media and culture conditions

M. tuberculosis was grown on Lowenstein-Jensen slants (Balows *et al.*, 1991a) at 37°C for three weeks. Organisms were scraped off and resuspended in Sauton's broth, a chemically defined medium (Atlas, 1993) to a density equivalent to a McFarland suspension of 1.0 (Balows *et al.*, 1991b). Sauton's broth (10 L) was inoculated with 0.1 volumes of culture suspension and incubated at 37°C for 4 weeks with shaking at 100 rpm until an absorbance at 600 nm of 0.29.

II.4. Assay for aminopeptidase

Aminopeptidase was assayed using ala-ala-phe-alpha-methylcoumarin (AAF-AMC; Sigma, St. Louis, MO, USA; Zimmerman *et al.*, 1977) as a substrate. The substrate was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg mL^{-1} and then diluted 1/ 5000 in phosphate buffered saline (PBS) pH 7.4 (Appendix 3) for use in the assay. Ten µl of enzyme solution to be assayed was pipetted into a 96-well polysorp nunc-immuno plate (Nalge Nunc International, Rochester, NY, USA) and 190 µl of assay solution was added. The fluorescence of the AMC released by the enzymatic hydrolysis of the substrate was read in an FL500 fluorescence plate reader (BioTek Instruments Inc., Winooski, VT, USA) with an excitation wavelength of 360 nm, an emission wavelength of 460 nm and a sensitivity

of 32. Assays were performed taking readings every minute for ten minutes or every fifteen minutes for one hour, depending on enzyme concentration.

II.5. Preparation of crude cell extracts

II.5.a. Preparation of extracellular filtrate concentrate (ECF)

Supernatant from cultures of *M. tuberculosis* H37Ra was harvested by removal of whole cells by centrifugation at 4000 x g for 10 min at 4°C. It was filter sterilized through a 0.22 micron filter and then concentrated 40-fold by a Miniplate™ Bioconcentrator (Amicon Inc., Beverly, MA, USA).

II.5.b. Preparation of sonicate

Whole cells isolated by centrifugation were used for the production of whole cell lysates or sonicate. Cells from 50 mL of 4 week old cultures were resuspended in 1 mL of lysis buffer. The cell suspension was transferred to 1 mL, o-ring sealed eppendorf centrifuge vials containing a 0.25 mL volume of silanized glass beads (425 - 600 micron, Sigma, St. Louis, MO, USA). The cell suspension was smeared and stained by the Ziehl-Neelsen method for mycobacteria (Balows *et al.*, 1991c) before and after sonication tc ensure purity and to estimate the degree of cell lysis. The vials were placed in a sonicating water bath until greater than 90 % cell lysis was observed by staining. The temperature of the water bath was maintained at 0°C by the addition of ice during sonication. Cell lysis routinely required 4 hours by this procedure. After lysis, cell debris was removed by centrifugation at 15,000 x g for 10 min at 4°C. A second lysis step was then performed by adding an additional 0.5 mL aliquot of lysis buffer to each tube, mixing by inversion and sonicating briefly. The mixture was centrifuged to separate cell debris and the supernatant was harvested and pooled. Finally, sonicate was filter sterilized through a 0.22 micron syringe top filter.

II.6. Purification of aminopeptidase

The fast performance liquid chromatography (FPLC) Basic System (Pharmacia

Biotech, Uppsala, Sweden) was used for column chromatography. The HILoad Q Sepharose Fast Flow column (26 mm x 10 cm) for anion exchange chromatography Phenyl Sepharose 6FF (high sub) column (0.7 mm x 2.4 cm) for hydrophobic interaction chromatography and the Superdex 200 HR 10/30 gel filtration column (10 mm x 30 cm) for size exclusion chromatography (Pharmacia Biotech, Uppsala, Sweden) were used. The A280, conductivity, and aminopeptidase activity were measured on all fractions and SDS-PAGE analysis of fractions of interest was performed after each purification procedure. Fractions containing activity were pooled for further purification. Aminopeptidase activity, absorbances at 280 and 210 nm and protein concentration using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) were measured on the pooled fractions from each column chromatography separation.

II.6.a. Q Sepharose Fast Flow anion exchange chromatography

ECF (219.5 mL) was loaded at 5 mL min^{-1} on the Q Sepharose Fast Flow column, which had been pre-equilibrated with 20 mM Bis-tris pH 6.5 and 5 mL fractions were collected. After washing with 3 column volumes of 20 mM Bis-tris pH 6.5 at 10 mL min^{-1}, baseline absorbance at 280 nm returned to 0 and protein was eluted with a 350 mL linear gradient from 0.0 M to 0.7 M NaCl in 20 mM Bis-tris pH 6.5 at 5 mL min^{-1}. The column was washed with 3 column volumes of 20 mM Bis-tris pH 6.5, 1M NaCl at 10 mL min^{-1}.

II.6.b. Phenyl HI SUB hydrophobic interaction chromatography

A 2 M ammonium sulfate solution (7.5 mL) was added to 22.5 mL of the pool containing maximum activity drop wise with stirring to an ammonium sulfate concentration of 0.5 M. The protein suspension was centrifuged at 20,000 x g for 30 min at 4°C to remove any precipitate. The resulting protein solution was loaded on the 1 mL Phenyl HI SUB column that had been pre-equilibrated with 50 mM sodium

phosphate pH 7.0, 0.5 M ammonium sulfate. The column was run at 1 mL min^{-1} collecting 2 mL fractions. The column was washed with 10 column volumes of start buffer and then a 50 mL gradient was run from 0.5 M to 0.0 M ammonium sulfate in 50 mM sodium phosphate pH 7.0. The column was washed with 10 column volumes of 50 mM sodium phosphate pH 7.0. The Phenyl HI SUB column pool (14 mL) was concentrated down to 1.0 mL by microconcentration in a centricon 10 (Amicon Inc., Beverly, MA, USA) at 5,000 x g at 4°C.

II.6.c. Superdex 200 size exclusion chromatography

The Phenyl HI SUB pool concentrate (0.5 mL) was loaded on the Superdex 200 column, which had been pre-equilibrated with 50 mM sodium phosphate pH 7.0, 0.15 M NaCl. The same buffer was pumped through the column at 0.25 mL min^{-1} and 0.5 mL fractions were collected.

II.6.d. Preparative SDS-PAGE and electroelution

The fractions with maximum activity were pooled and 850 µl was combined with 170 µl of 6 x Laemmli's digestion mix (LDM) and loaded on a 1.5 mm thick 12.5 % SDS-polyacrylamide gel without boiling the samples prior to loading. The gel was run with constant current of 12 milliamps at 10°C. The gel was sliced horizontally into 2 mm widths and each slice was electroeluted in a Little Blue Tank multifunction electrophoresis system (ISCO, Inc., Lincoln, NE, USA). Electroelution was performed at 150 volts for 4 hours in a cold room (4°C) using the small traps, which produce a final volume of 75 µl. Gel slices were placed on top of plastic screens above the trap and a dialysis membrane with a molecular weight cut off of 6000 - 8000 daltons (Spectra/Por, Sigma, St. Louis, MO, USA) was used on each side of the trap. Electroelution tank buffer was 1 x SDS-PAGE running buffer and the trap buffer used was 0.1 x SDS-PAGE running buffer. One third of each sample was used for SDS-PAGE analysis. The remainder of the samples were diluted to 1.0 mL in PBS

pH 7.4 and dialyzed using membranes with a molecular weight cut off of 6000 - 8000 daltons at 4ºC against three changes of 1000 volumes of PBS pH 7.4 containing 0.1 mM $ZnCl_2$ for 8 hours each.

II.7. Analytical SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970) under reducing conditions using an SE600 protein gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). Gels were 1.5 mm thick and contained 12.5 % polyacrylamide in the separating gel. Protein samples were mixed 5:1 with 6 x LDM and heated to 95ºC for 5 min prior to loading. Standards used were Bio-Rad's low molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) diluted 1 in 100 with 1 x LDM. Standards include: phosphorylase b (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400). Gels were electrophoresed for 16 hours at a constant current of 20 milliamps at 10ºC and stained with Bio-Rad's Silver Stain Kit (Bio-Rad Laboratories, Hercules, CA, USA).

II.8. Characterization of the purified protein

II.8.a. Determination of the molecular weight of the native purified protease

Estimation of the molecular weight of the native aminopeptidase was made by gel filtration chromatography on the Superdex 200 column. A standard curve of the log of the molecular weight versus the elution volume was produced using 6 molecular weight standards. Molecular weight standards used were: blue dextran (Mr 2,000,000), beta-amylase (Mr 200,000), alcohol dehydrogenase (Mr 150,000), bovine serum albumin (Mr 66,000), carbonic anhydrase (Mr 29,000) and cytochrome c (Mr 12,400; Sigma, St. Louis, MO, USA). Standards (100 µg of each protein in a total volume of 0.5 mL) were loaded on the column, which had been pre-equilibriated with 50 mM sodium phosphate pH 7.0, 0.15 M NaCl. The column was run at

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II.9.b. Effect of temperature on aminopeptidase activity

ECF or purified aminopeptidase was added to substrate that had been pre-equilibriated to a temperature in the range of 20°C - 65°C to evaluate the effect of temperature on enzyme activity. Aliquots (200 µL) were removed at times from 0 to 30 min and a single reading of the fluorescence of the mixture was taken at each time point.

II.9.c. Inhibition of aminopeptidase activity

The activity of concentrated ECF and purified aminopeptidase were evaluated in the presence of protease inhibitors at the following concentrations; amastatin (10 µM), bestatin (10 µM), 3,4-dichloroisocoumarin (3,4-DCI, 0.1 mM), E-64 (10 µM), EDTA (10 mM), leupeptin (0.1 mM), pepstatin (1 µM), 1,10-phenanthroline (1.0 mM), PMSF (1 mM) and Zincov™ (0.1 mM). All inhibitors were purchased from Sigma (St. Louis, MO, USA) except for Zincov™ (Calbiochem-Novabiochem Corp, La Jolla, CA, USA). The enzyme was pre-incubated with inhibitor or, as a control, with an amount of solvent equivalent to that used to dissolve the inhibitor; methanol for amastatin, bestatin, PMSF and 1,10-phenanthroline, and DMSO for 3,4-DCI and pepstatin, for 30 min. E-64, EDTA, leupeptin and Zincov™ were dissolved in water. The assay was initiated by the addition of substrate and assays were performed as described above (section II.4).

II.9.d. Effect of metal ions on restoration of aminopeptidase activity

EDTA was added to concentrated ECF and purified protease to a final concentration of 10 mM. The mixture was incubated at 21°C for 30 min and then assayed to ensure that no enzyme activity was present. Each mixture was then precipitated by adding solid ammonium sulfate to a final concentration of 60 % at 4°C. The mixtures were centrifuged for 10 min at 10,000 x g at 4°C to separate the protease from the EDTA. Dilutions of $ZnCl_2$, $MgCl_2$ and $CaCl_2$ ranging from 5 µM

to 10 mM were incubated with both resuspended ECF or purified protease. Activity was assayed as described above (section II.4).

II.9.e. Determination of substrate specificity of aminopeptidase

Aminopeptidase activity of both ECF and purified protease was assayed using the following substrates: N-succinyl–GPLGP-AMC, N-succinyl-AAF-AMC, AAF-AMC, A-AMC, R-AMC, E-AMC, G-AMC, I-AMC, L-AMC, F-AMC, P-AMC, S-AMC and V-AMC. Substrates were dissolved in DMSO to a concentration of 0.1075 M and then diluted 1/ 5000 in PBS pH 7.4 for use in the assay (final concentration = 2.15×10^{-5} M) as described previously in this Chapter for AAF-AMC (section II.4).

Km, Vmax and turnover rate were determined for purified aminopeptidase activity against the substrates R-AMC, L-AMC, and P-AMC. Substrates were diluted in PBS pH 7.0 to the following concentrations: 0.5 μM, 1.0 μM, 1.5 μM, 2.0 μM, 2.5 μM, 3.0 μM, 4.0 μM, 6.0 μM, 8.0 μM, 10.0 μM, 20.0 μM and 40.0 μM. The assay was performed at 32°C. The initial velocity for each substrate was determined at each concentration with the substrate remaining in excess of the maximum fluorescence obtained by at least 10-fold. Km and Vmax were read from a direct linear plot. Each experiment was performed in triplicate.

II.10.a. N-terminal amino acid sequencing

The protein band to be sequenced was run on SDS-PAGE as described earlier (section II.7) with modifications described by Ursitti *et al.* (1997). Briefly, the gel was cast the day before it was used and allowed to completely polymerize for 24 hours at 21°C; only one half of the usual amount of ammonium persulfate was added to the stacking gel (0.0375 % final concentration in the gel mix); gels were pre-run for 15 min before sample was loaded; and samples were not boiled, but rather heated to 37°C for 15 min prior to loading. Three lanes containing the purified protein were

blotted onto a 0.2 micron polyvinylidene difluoride immobilon membrane (Bio-Rad Laboratories, Hercules, CA, USA) in a TE series Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The buffer used for transfer was 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS), 10 % methanol pH 11.0 and transfer was performed at 25 volts for 16 hours at 4°C. After rinsing the membrane well with milliQ quality water, it was stained for 1 minute in 0.2 % Ponceau S in 3 % w/v trichloroacetic to visualize the bands. The bands on the membrane were cut out and air-dried for 30 minutes. Sequencing was performed by Edman degradation on a Hewlitt Packard HP1005A and detection of phenylthiohydantoin-amino acids was performed by reverse phase-high pressure liquid chromatography reading absorbance at 269 nm at the Alberta Peptide Institute (Edmonton, Alberta, Canada).

II.10.b. Amino acid sequence similarity search

A search of the N-terminal sequence derived for the purified protein was performed in the Genbank database using the BLAST algorithm (Altschul *et al.*, 1990). The accession number of the closely matching *Mycobacterium tuberculosis* strain H37Rv putative aminopeptidase is AL021246. The accession number of the *Streptomyces lividans* 66 *pepN* gene product is L23172.

II.11. Determination of SDS concentration

The amount of SDS present after electroelution of the protease and after dialysis of the product was determined as described by Waite and Wang (1976). A 1.0 mM solution of SDS was used to generate a standard curve. In triplicate; 0, 20, 40, 60, 80 and 100 µL of solution was pipetted into 10 mL glass culture tubes resulting in 0, 20, 40, 60, 80 and 100 nmoles of SDS in each tube, respectively. Samples (100 µL) of purified protease from electroelution before and after dialysis were also added to culture tubes in triplicate. The volume in each tube was increased

to 1.0 mL by addition of milliQ water. The following were added to each tube: 1.0 mL of 0.03 M HCl, 1.0 mL of basic fuschin (48 mg mL^{-1} in milliQ water; Sigma Chemical Co., St. Louis, MO, USA) and 2.0 mL of chloroform. Tubes were mixed thoroughly and then incubated in a preheated 60°C water bath for 15 min. Samples were removed, mixed well again and cooled to room temperature (21°C) for 1 hour. The upper, aqueous phase was aspirated off and the absorbance of the lower chloroform phase containing the dodecyl sulfate-basic fuschin complex was read at 553 nm in an Ultrospec 3000 (Pharmacia Biotech, Uppsala, Sweden). A standard curve was produced plotting the absorbance at 553 nm versus the nmoles of SDS added to the tubes. The amount of SDS present in the samples tested was determined from the standard curve.

II.12. Western immunoblotting

Kaleidoscope prestained molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA), son, ECF and purified protease were run on a 12.5 % SDS-polyacrylamide gel in duplicate. Proteins from SDS-PAGE gels were transferred to trans-blot transfer medium (0.45 micron nitrocellulose membrane; Bio-Rad Laboratories, Hercules, CA, USA) by the method of Towbin *et al.* (1979). Electrophoretic transfer was performed in Towbin buffer at 250 milliamps for 3 hours at 4°C in a TE series Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA).

Buffers and solutions used during immunodetection were provided in the Immuno-blot assay kit, goat anti-human IgG (H+L) alkaline phosphatase from Bio-Rad Laboratories (Hercules, CA, USA). The sources of primary antibody were sera from two patients diagnosed with active tuberculosis. Primary antibody was incubated with the blots at a concentration of 2 % for 2 hours. All other steps in immunoblotting were as described in the Bio-Rad Immuoblot assay kit. The secondary antibody was

alkaline phosphatase coupled to goat anti-human IgG. Colorimetric detection was performed by adding 1 mL of 50 mg mL^{-1} *p*-nitro blue tetrazolium chloride (NBT) in 70 % N,N-dimethylformamide (DMF) and 1 mL of 25 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) in DMF to 48 mL of carbonate buffer pH 9.8. Color development proceeded for 15 min and was stopped by rinsing the blot in distilled water.

Chapter III. Results

III.1. *Mycobacterium tuberculosis* growth and aminopeptidase production

The growth of *M. tuberculosis* was measured weekly for 6 weeks by reading the A600 of the culture. Extracellular filtrate concentrate (ECF) and sonicate were prepared from an aliquot of the culture at each time point and assayed for the amount of aminopeptidase activity present (FIG. III-1a). Activity was detected in both ECF and sonicate at 7 days post inoculation. The amount of activity in sonicate increased for 6 weeks but increased more slowly after 4 weeks. The amount of activity present in ECF increased for 6 weeks with a larger increase in the amount detected at 4, 5 and 6 weeks. The amount of activity per culture growth, estimated from the A600 of the culture at each time point, is shown in FIG. III-1b. The amount of activity in sonicate per culture growth increased slowly for the first 3 weeks and then drops and leveled off at a lower value for weeks 4, 5 and 6. The amount of aminopeptidase activity in ECF increased during weeks 1, 2 and 3 but the increase was greater in weeks 4, 5 and 6.

III.2. Purification of *M. tuberculosis* aminopeptidase

Aminopeptidase activity was purified more than 30, 000 fold from 4 week ECF with a net yield of 25 % of enzyme activity (Table III-1).

III.2.a. Q Sepharose Fast Flow anion exchange chromatography

The purification achieved by the Q Sepharose Fast Flow column was more than 60 fold with a yield of 87 % of activity (Table III-1). The peak of aminopeptidase activity eluted at a salt concentration of 0.34 M NaCl in 20 mM Bis-tris pH 6.5. The salt concentration of the fractions with activity that ranged from 0.32

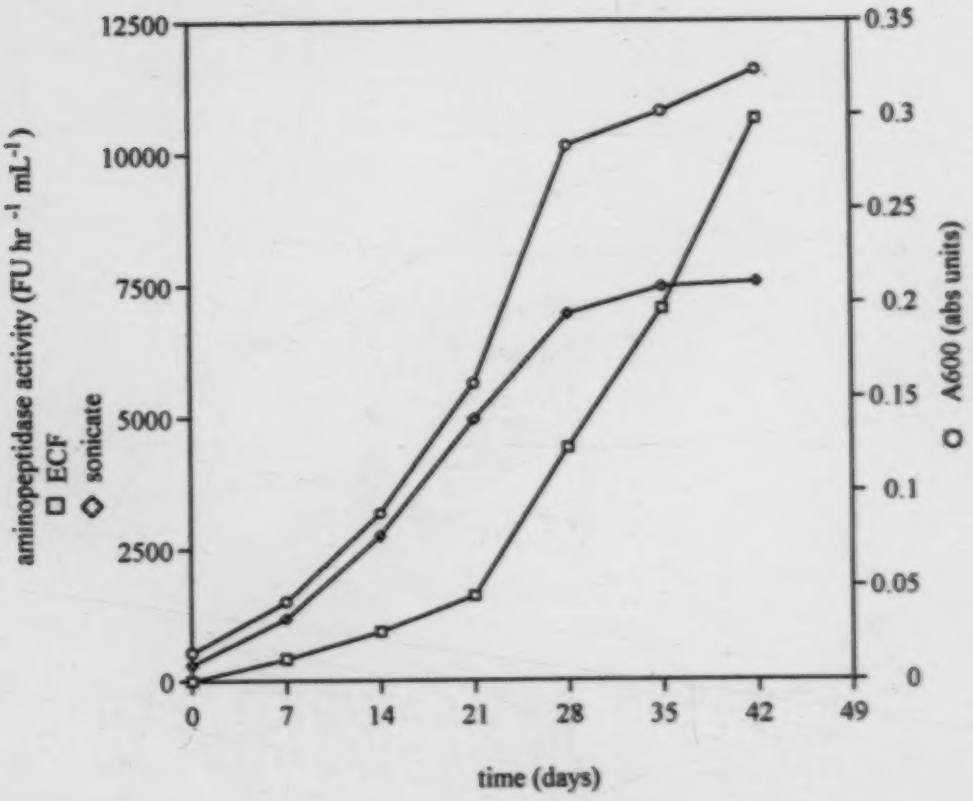


FIG. III-1a. Amount of aminopeptidase activity in extracellular filtrate (ECF) and sonicate produced from 1 mL of culture measured at weekly intervals for 6 weeks. Activity was measured by reading the fluorescence produced upon digestion of the synthetic substrate AAF- α-methylcoumarin (AAF-AMC). The number of cells present was estimated by reading the A600 (absorbance at 600 nm) of the culture at each time point. 1 FU = 0.34 pmoles AMC.

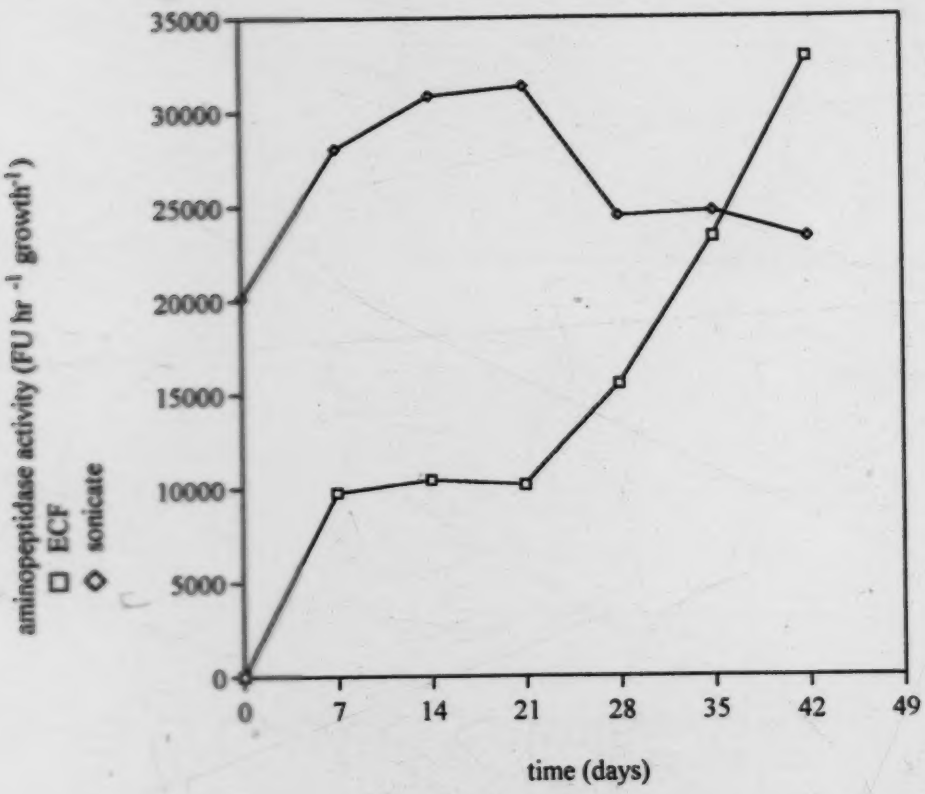


FIG. III-1b. Amount of aminopeptidase in extracellular filtrate (ECF) and sonicate per culture growth measured at weekly intervals for 6 weeks. Activity was measured by reading the fluorescence produced upon digestion of the synthetic substrate AAF- α-methylcoumarin (AAF-AMC). The culture growth was estimated by reading the A600 (absorbance at 600 nm) of the culture at each time point. 1 FU = 0.34 pmoles AMC.

TABLE III-1. Summary of aminopeptidase purification

sample[a]	protein (mg mL^{-1})[b]	specific activity (FU hr^{-1} mg^{-1})[c]	net purification (fold)	net yield (%)
conc ECF	2.06	106,553	-	100.0
Q Seph FF	0.30	6,780,000	63.6	87.1
Phe HI SUB	0.520	53,100,000	499	67.6
Superdex 200	0.063	137,000,000	1,280	42.2
SDS-PAGE	0.006	3,380,000,000	31,800	24.5

[a] Abbreviations: conc ECF, concentrated extracellular filtrate; Q Seph FF, anion exchange chromatography on a Q Sepharose Fast Flow column; Phe HI SUB, hydrophobic interaction chromatography on a high substituted phenyl sepharose column; Superdex 200, size exclusion chromatography on a Superdex 200 column; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis. Samples are the product of the purification procedure listed.

[b] Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA, USA).

[c] Aminopeptidase activity was assayed by measuring the increase in fluorescence in one hour due to the release of α-methylcoumarin from the synthetic substrate, AAF-AMC as described in section II.3. 1 FU = 0.34 pmoles AMC.

– 0.36 M NaCl were pooled (FIG. III-2). Pooled fractions which contained more than 20 % of the peak of activity yielded a volume of 25 mL. The protease activity was concentrated 9.3 fold over the initial material loaded (Table III-1). FIG. III-3 shows a silver stained SDS-PAGE of the fractions of interest through the elution procedure. There was a large amount of protein which did not adhere to the column (lane 3), washed off in the starting buffer (0.0 M NaCl; lane 4) or washed off in the final wash buffer (1.0 M NaCl; lane 10). There is a band at the molecular weight of the purified aminopeptidase visible in lanes 6 – 9 which corresponds with the elution of aminopeptidase activity, (peak is in lane 7; FIG. III-3).

III.2.b. Phenyl HI SUB hydrophobic interaction chromatography

The purification achieved by the Phenyl HI SUB column was approximately 8 fold with a yield of 77 % of activity. The peak of aminopeptidase activity eluted at a salt concentration of 0.12 M ammonium sulfate in 50 mM sodium phosphate pH 7.0. The salt concentration of the fractions pooled for activity ranged from 0.22 – 0.06 M ammonium sulfate (FIG. III-4). Pooled fractions which contained more than 10 % of the peak of activity yielded a volume of 18 mL. The protease activity was concentrated 13.5 fold over the initial material loaded (Table III-1). FIG. III-5 shows a silver stained SDS-PAGE of the fractions of interest through the elution procedure. There was some protein which did not adhere to the column (lane 3), washed off in the starting buffer (0.5 M ammonium sulfate; lane 4) or which washed off in the final wash buffer (0.0 M ammonium sulfate; lane 10). There is a band at the molecular weight of the purified aminopeptidase visible in lanes 5 – 9 which corresponds with the elution of aminopeptidase activity (peak is in lane 7; FIG. III-5).

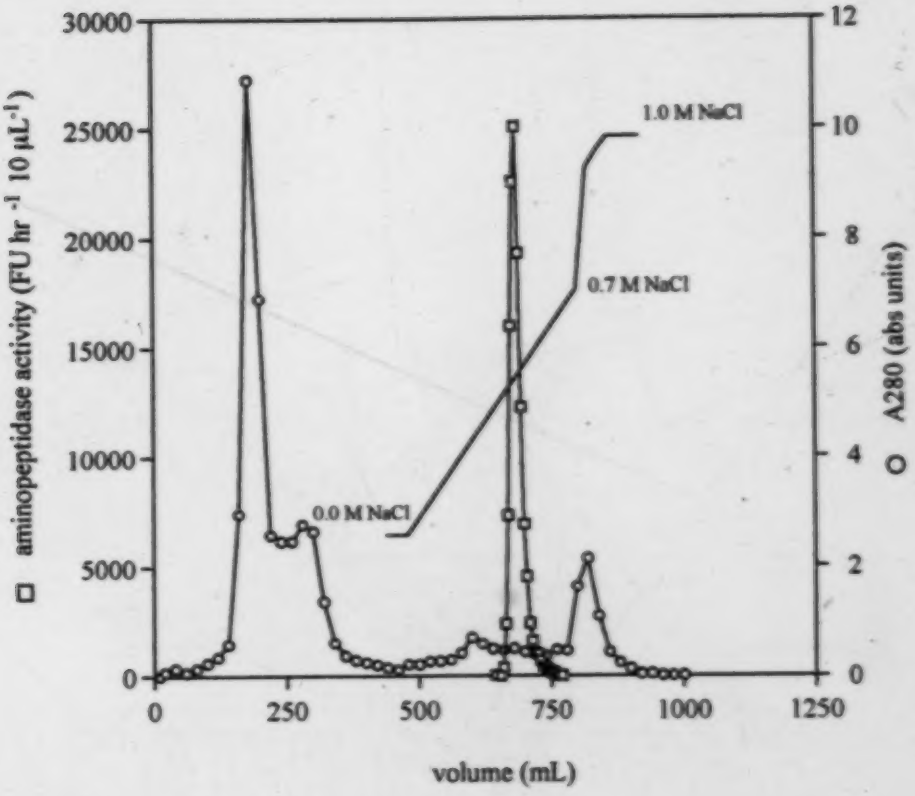


FIG. III-2. Profile of aminopeptidase elution from the Q Sepharose Fast Flow anion exchange chromatography column. The peak of protease activity eluted at a salt concentration of 0.34 M NaCl in 20 mM Bis-tris pH 6.5. Salt concentration in fractions was determined by measuring the conductivity of fractions and is shown as a solid line on the graph. 1 FU = 0.34 pmoles AMC released from AAF-AMC; A280 = absorbance at 280 nm.

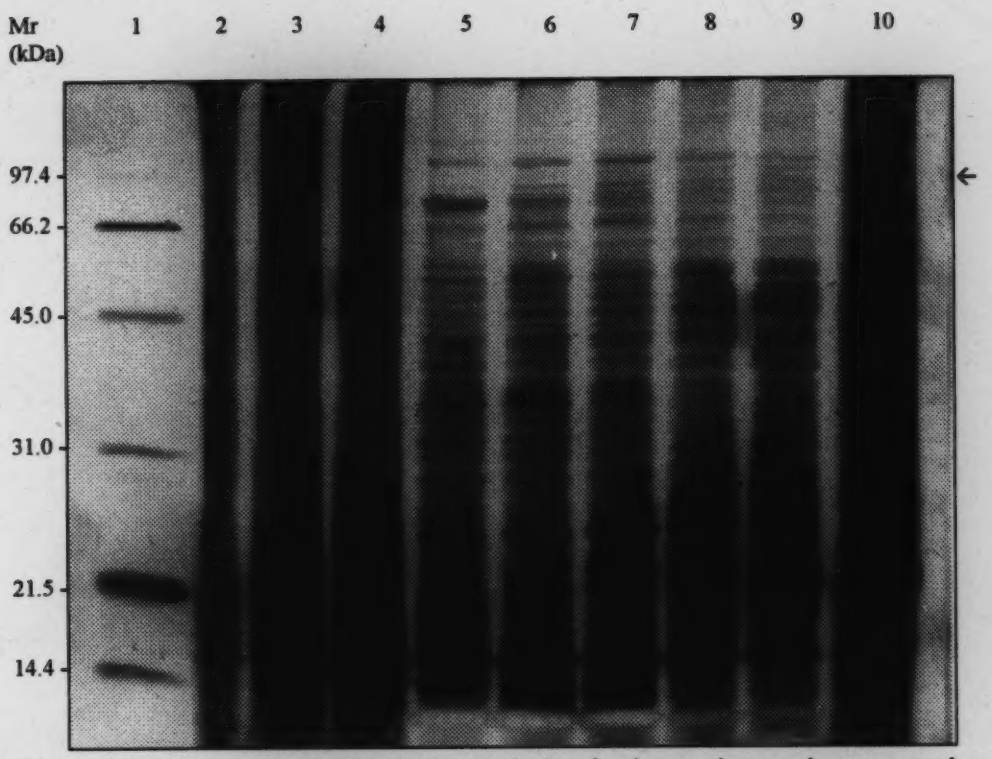


FIG. III-3. Silver stained SDS-PAGE analysis of anion exchange chromatography column fractions. Low molecular weight markers: phosphorylase b (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400; lane 1). ECF (50 μL, lane 2). Non-adherent loaded material (150 μL, lane 3). 0.0 M NaCl column wash (150 μL, lane 4). 0.29 M NaCl column fraction (150 μL, lane 5). 0.32 M NaCl column fraction (150 μL, lane 6). 0.34 M NaCl column fraction (150 μL, lane 7). 0.37 M NaCl column fraction (150 μL, lane 8). 0.40 M NaCl column fraction (150 μL, lane 9). Final 1.0 M NaCl column wash (150 μL, lane 10). Mr = molecular weight; kDa = kilodaltons. The peak of aminopeptidase activity eluted at a salt concentration of 0.34 M NaCl (lane 7). The arrow shows the position of the purified aminopeptidase on SDS-PAGE.

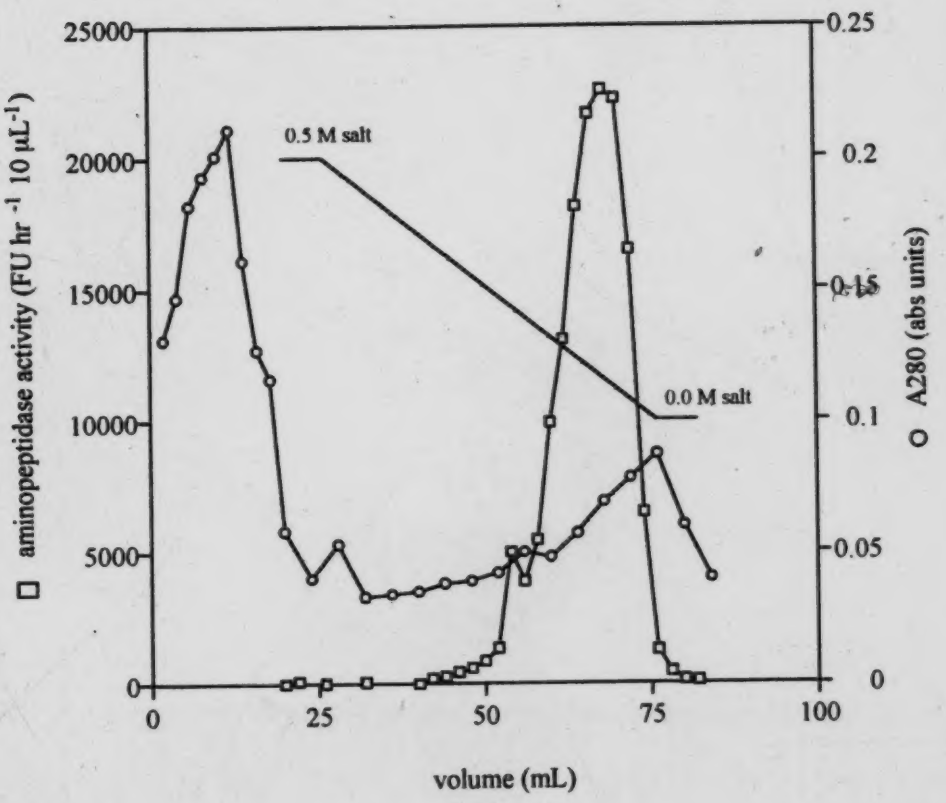


FIG. III-4. Profile of aminopeptidase elution from Phenyl HI SUB hydrophobic interaction chromatography column. The peak of protease activity eluted at a concentration of .12 M ammonium sulfate in 50 mM sodium phosphate pH 7.0. Salt concentration in the fractions was determined by measuring conductivity of fractions and is shown as a solid line on the graph. 1 FU = 0.34 pmoles AMC released from the synthetic substrate, AAF-AMC; A280 = absorbance at 280 nm.

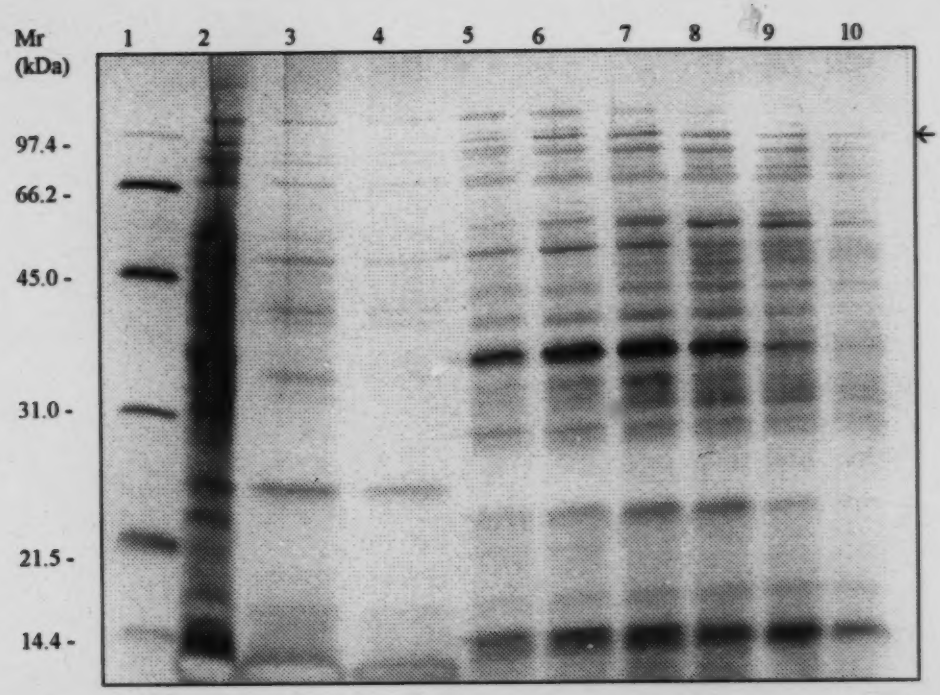


FIG. III-5. Silver stained SDS-PAGE analysis of hydrophobic interaction chromatography column fractions. Low molecular weight markers: phosphorylase b (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400; lane 1). Q Sepharose Fast Flow column pool (150 µL, lane 2). Non-adherent loaded material (150 µL, lane 3). 0.5 M salt column wash (150 µL, lane 4). 0.24 M salt column fraction (150 µL, lane 5). 0.18 M salt column fraction (150 µL, lane 6). 0.12 M salt column fraction (150 µL, lane 7). 0.08 M salt column fraction (150 µL, lane 8). 0.04 M salt fraction (150 µL, lane 9). 0.0 M salt column wash (150 µL, lane 10). Mr = molecular weight; kDa = kilodaltons. The peak of aminopeptidase activity eluted at a salt concentration of 0.12 M ammonium sulfate (lane 7). The arrow indicates the position of the purified protease by SDS-PAGE.

III.2.c. Non-denaturing Superdex 200 size exclusion chromatography column

The purification achieved by non-denaturing size exclusion chromatography was 2.6 fold with a yield of 62.6 % of activity (Table III-1). The peak of aminopeptidase activity eluted from the Superdex 200 column at a volume of 13.7 mL after loaded material entered the column. There were three 0.5 mL fractions which contained more than 10 % of the activity of the peak (FIG. III-6). SDS-PAGE analysis of column fractions revealed that some high molecular weight proteins which run very closely to the aminopeptidase on SDS-PAGE were removed by this step simplifying accurate excision of a single band from SDS-PAGE (FIG. III-7, lane 5). The peak of activity is present in the fraction represented in lane 6 (FIG. III-7).

III.2.d. Preparative SDS-PAGE and electroelution of the aminopeptidase

The purification achieved by electroelution was approximately 25 fold with a yield of 58 % (Table III-1). The purified aminopeptidase appeared as a single band on silver stained SDS-PAGE (FIG. III-8, lane 3). After electroelution of gel slices from SDS-PAGE and reconstitution of activity, aminopeptidase activity was found in only one gel slice.

III.2.e. Summary of purification

The purification is summarized in FIG. III-9 by showing the protein bands visible on a silver stained SDS-PAGE after each column purification and the final electroelution step. Table III-1 summarizes the purification and net yield of each step.

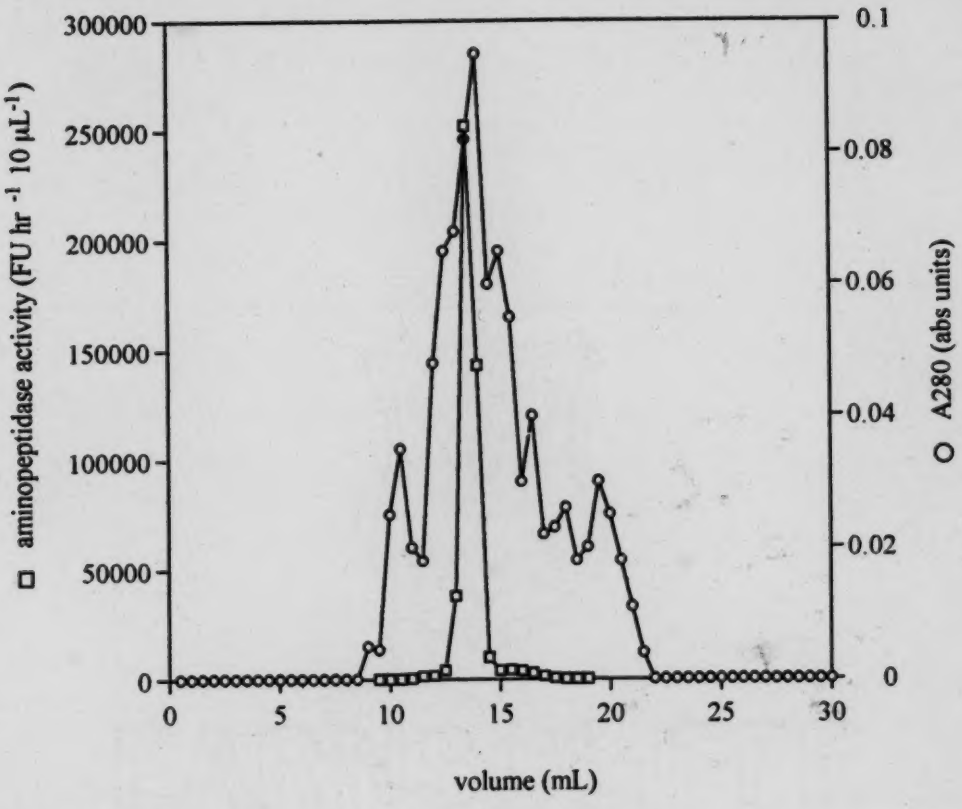


FIG. III-6. Profile of aminopeptidase elution from the Superdex 200 size exclusion chromatography column. 1 FU = 0.34 pmoles AMC released from the synthetic substrate, AAF-AMC; A280 = absorbance at 280 nm. The peak of aminopeptidase activity eluted at 13.7 ± .08 mL after injection of the Phe HI SUB column pool corresponding to a molecular weight of 80± 5 kilodaltons.

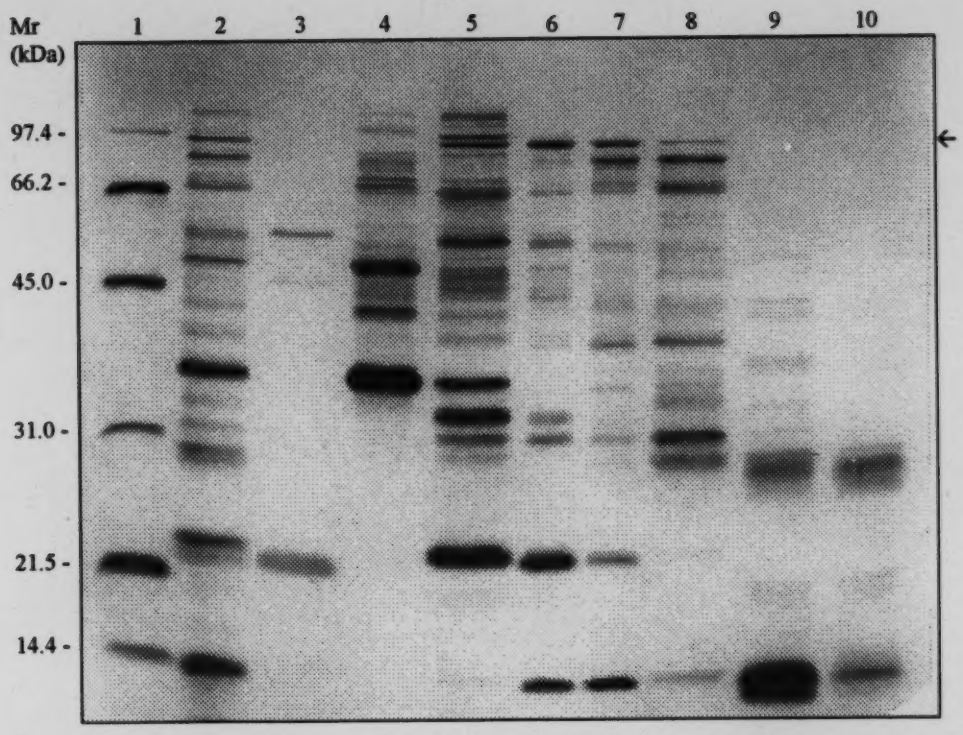


FIG. III-7. Silver stained SDS-PAGE analysis of Superdex 200 size exclusion chromatography column fractions. Low molecular weight markers: phosphorylase b (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400; lane 1). Phenyl HI SUB hydrophobic interaction chromatography column pool (150 µL, lane 2). Fractions are described as volumes at which they eluted after loading the Phenyl HI SUB column pool. 11.0 mL (150 µL, lane 3). 12.5 mL (150 µL, lane 4). 13.5 mL (150 µL, lane 5). 14.0 mL (50 µL, lane 6). 14.5 mL (50 µL, lane 7). 15.0 mL (150 µL, lane 8). 16.0 mL (150 µL, lane 9). 17.0 mL (150 µL, lane 10). Mr = molecular weight ; kDa = kilodaltons. The peak of aminopeptidase activity eluted at a volume of 13.7 $\pm$ .08 mL (lane 6) corresponding to a molecular weight of 80 $\pm$ 5 kDa. The arrow shows the location of the purified aminopeptidase on SDS-PAGE.

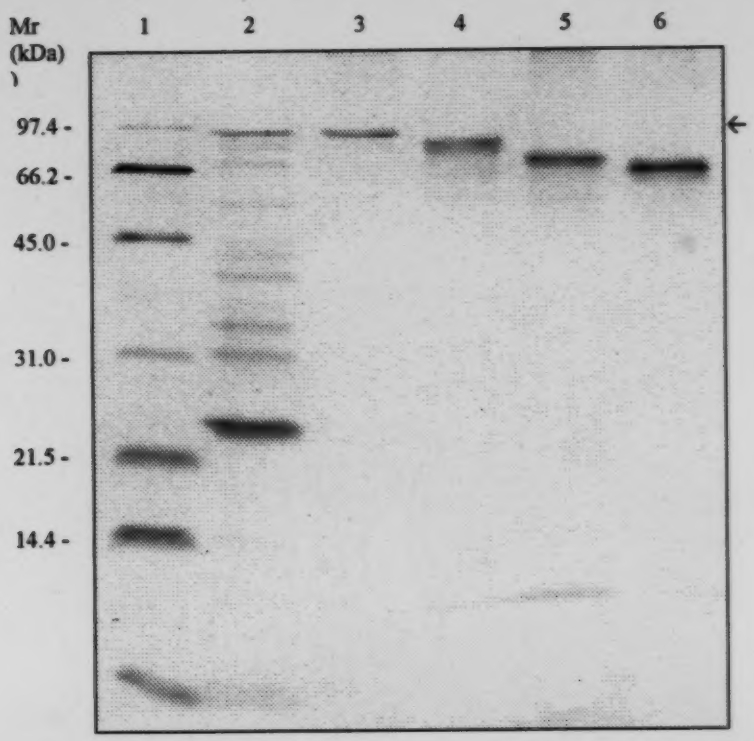


FIG. III-8. SDS-PAGE analysis of electroeluted gel slices excised from a preparative gel of the Superdex 200 column pool. Low molecular weight markers: phosphorylase b (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400; lane 1). Superdex 200 column pool (50 μL, lane 2). Electroeluted gel slice 1 (50 μL, lane 3). Electroeluted gel slice 2 (150 μL, lane 4). Electroeluted gel slice 3 (150 μL, lane 5). Electroeluted gel slice 4 (150 μL, lane 6). Mr = molecular weight; kDa = kilodaltons. Aminopeptidase activity was recovered in gel slice 1 (lane 3) after dialysis in three changes of PBS pH 7.4 with 1 mM zinc chloride for 24 hours. The arrow shows the position of the purified aminopeptidase.

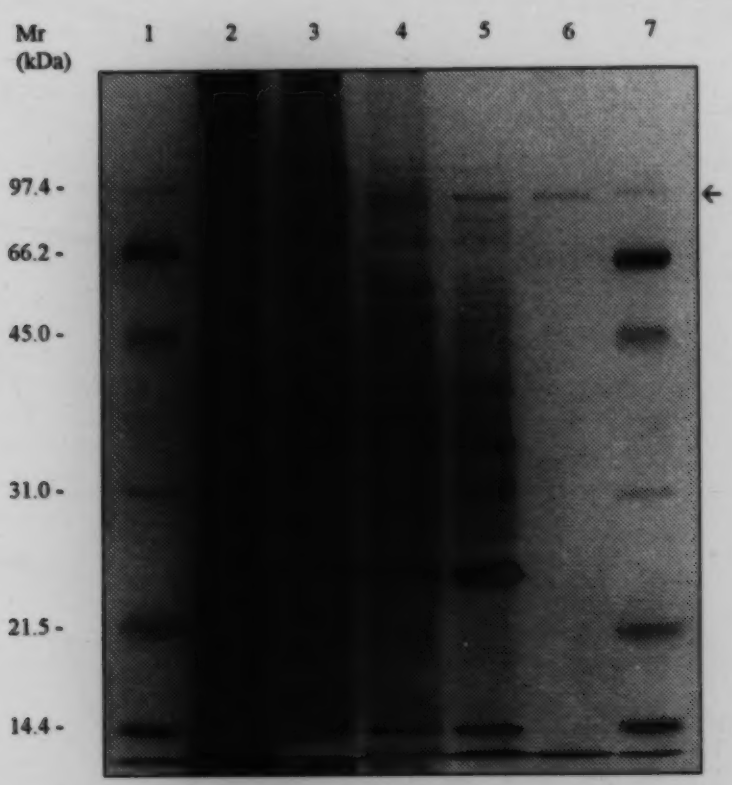


FIG. III-9. Silver stained SDS-PAGE analysis of steps in purification of aminopeptidase from extracellular filtrate (ECF). Low molecular weight markers: phosphorylase b (Mr 97,400), bovine serum albumin (Mr 6,200), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400; lanes 1 and 7). ECF (206 μg, lane 2). Q Sepharose Fast Flow column pool (45.0 μg, lane 3). Phenyl HI SUB column pool (16.4 μg, lane 4). Superdex 200 column pool (6.3 μg, lane 5). Electroeluted gel slice (0.3 μg, lane 6). Mr = molecular weight; kDa = kilodaltons.

III.3. Characterization of the purified protein

III.3.a. Determination of the molecular weight of the non-denatured protein by Superdex 200 size exclusion chromatography

FIG. III-10 shows the linear relationship of the log of the molecular weight of known molecular weight standards versus their elution volume from the Superdex column separation run under the same, non-denaturing conditions as the aminopeptidase. The purified protease eluted at a volume of 13.7 $\pm$ .08 mL from the Superdex 200 column corresponding to a molecular weight of approximately 80 $\pm$ 5 kDa from the standard curve.

III.3.b. Determination of the molecular weight of the denatured protein by SDS-PAGE

FIG. III-11 shows the linear relationship between the log of the molecular weight of known molecular weight markers versus their relative migration (Rf) on SDS-PAGE. Relative migration is the distance that a protein band traveled divided by the distance traveled by the running front of the gel. The aminopeptidase Rf value corresponds to a molecular weight of 93 kDa.

III.3.c. Determination of the isoelectric point of the purified aminopeptidase

The isoelectric point of the aminopeptidase was determined using a chromatofocusing column and eluting with polybuffers diluted 1 in 10 ranging from a pH of 7.4 to 3.0. (FIG. III-12). The pI of this protein by chromatofocusing was 4.1 $\pm$ 0.1.

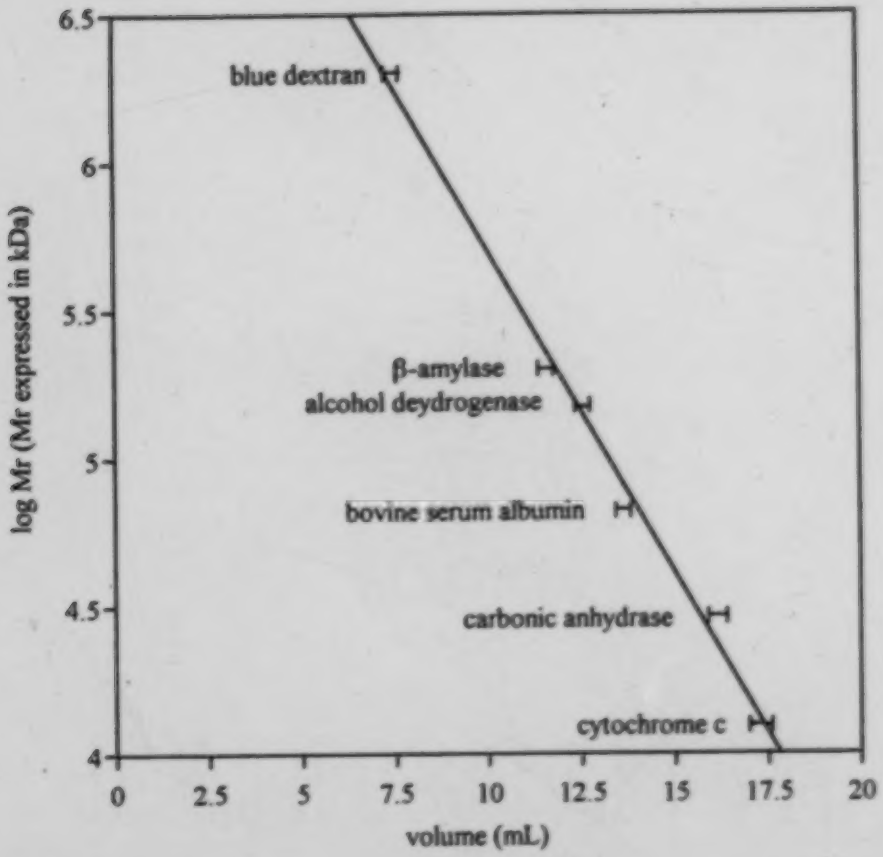


FIG. III-10. Standard curve of the elution of molecular weight markers from non-denaturing Superdex 200 size exclusion chromatography column. Markers include: blue dextran (Mr 2,000 kDa), β-amylase (Mr 200 kDa), alcohol dehydrogenase (Mr 150 kDa), bovine serum albumin (Mr 66 kDa), carbonic anhydrase (Mr 29 kDa) and cytochrome c (Mr 12.4 kDa; Sigma Chemical Co., St. Louis, MO, USA). The aminopeptidase activity peak eluted at 13.7 ± .08 mL corresponding to a molecular weight of 80± 5 kDa. Mr = molecular weight; kDa = kilodaltons. Standard deviations are reported for n=3.

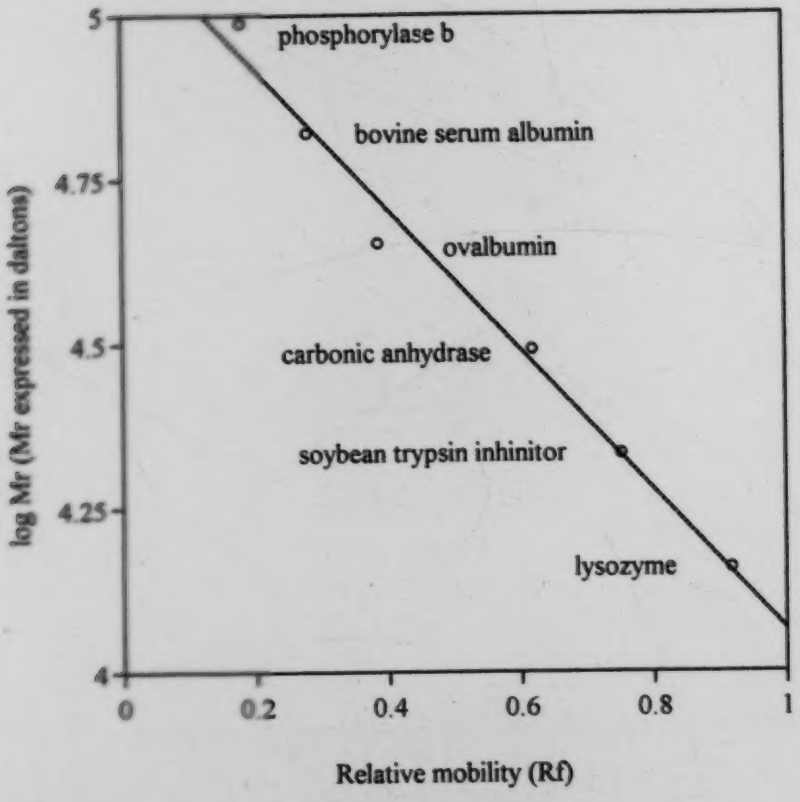


FIG. III-11. Standard curve of the migration of molecular weight markers on a 12.5% SDS-PAGE. Markers include: phosphorylase b (Mr 97.4 kDa), bovine serum albumin (Mr 66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (Mr 31 kDa), soybean trypsin inhibitor (Mr 21.5 kDa) and lysozyme (Mr 14.4 kDa; Bio-Rad Laboratories, Hercules, CA, USA). Mr = molecular weight; kDa = kilodaltons. The purified aminopeptidase migrated 22.8 mm (Rf = 0.19) which corresponds to a molecular weight of 93 kDa.

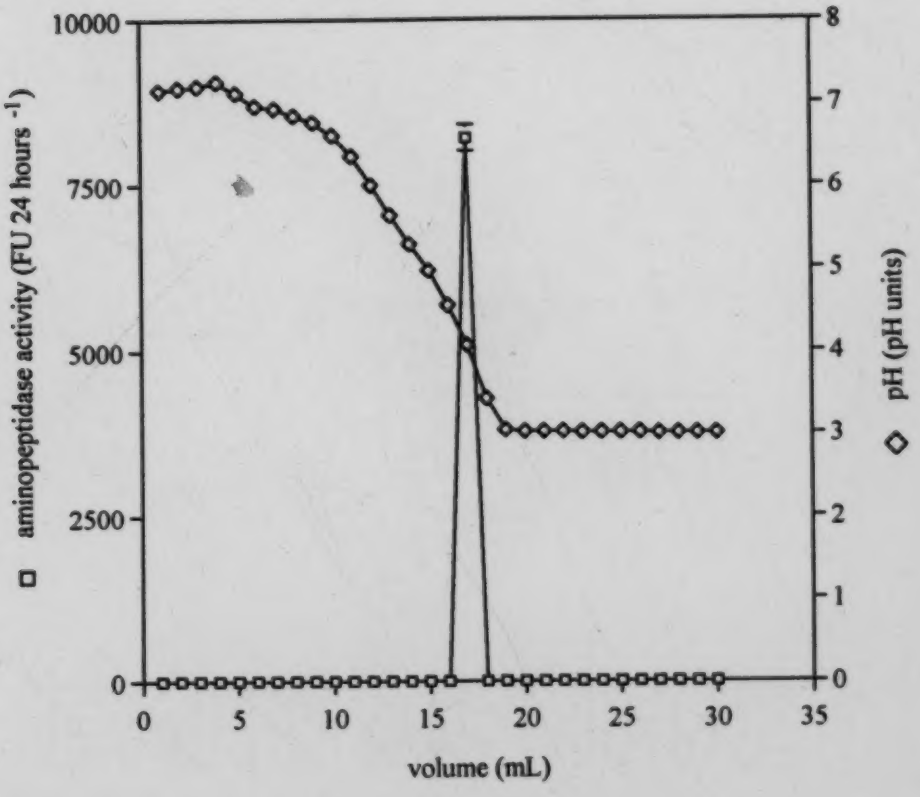


FIG. III-12. Determination of the isoelectric point of purified aminopeptidase. Purified protease (0.03 μg) was loaded onto a chromatofocusing column and eluted with a decreasing pH gradient as described in section II.8.c. 1 FU = 0.34 pmoles AMC released from the synthetic substrate, AAF- α-methylcoumarin (AAF-AMC). Standard deviations are calculated for n=3.

III.4. Characterization of the aminopeptidase activity

III.4.a. pH optimum of aminopeptidase activity

Aminopeptidase activity was optimal at a pH of 7.0 (FIG. III-13). It maintained more than 50 % optimal activity from pH 6.0 to 8.0 and still displayed detectable activity from pH 5.5 – 8.5. ECF and partially purified protease show similar pH optima for aminopeptidase activity.

III.4.b. Temperature optimum of aminopeptidase activity

The aminopeptidase temperature optimum was 32°C for both ECF and purified aminopeptidase (FIG. III-14). More than 20 % of activity was retained at 20°C and more than 30 % of activity was retained at 48°C. Activity was reduced at temperatures ≥ 56°C but 20 and 25 % of activity were restored for ECF and purified aminopeptidase, respectively, when the temperature was subsequently reduced to 32°C and re-assayed.

III.4.c. Inhibition of aminopeptidase activity

Aminopeptidase activity of ECF and purified protease was determined in the presence of protease inhibitors to determine the class of protease. Amastatin and bestatin, aminopeptidase inhibitors (Benyon and Salvesen, 1993), fully inhibited both ECF and purified aminopeptidase at 10 μM (Table III-2). Its aminopeptidase activity had been previously confirmed by the inability of the enzyme to digest N-terminally protected substrates (Table III-4; compare AAF-AMC to N-succ-AAF-AMC). Enzyme activity was inhibited by 10 mM EDTA and 1,10-phenanthroline, metallo-enzyme inhibitors. Finally, activity was inhibited by Zincov™, an inhibitor of members of the neutral, zinc-requiring metalloprotease family. Aminopeptidase

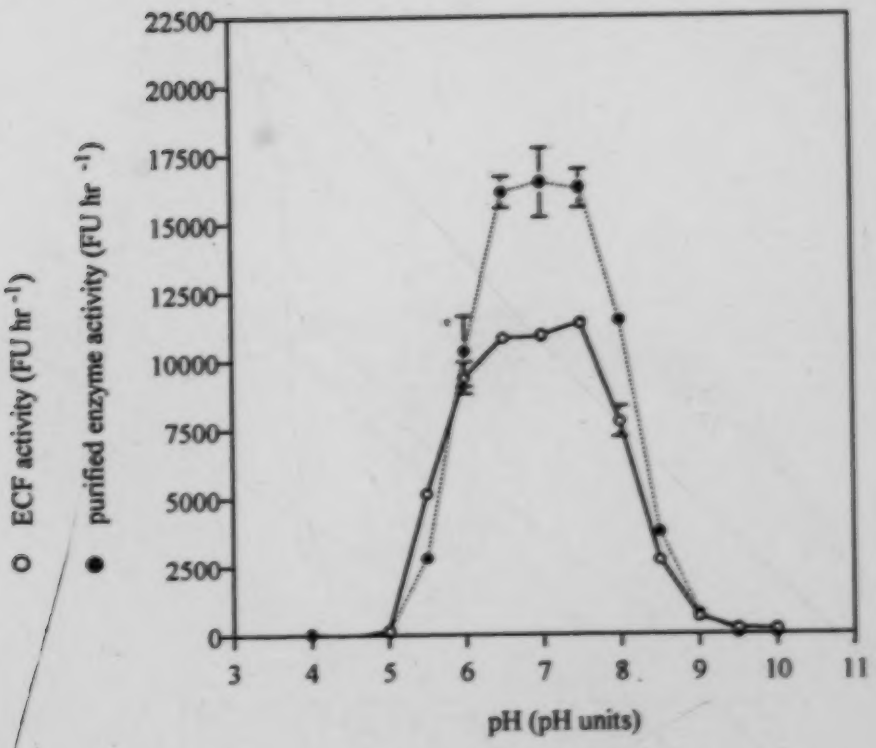


FIG. III-13. Effect of pH on aminopeptidase activity. Activity of extracellular filtrate (ECF; 0.1 mg) and purified protease (5 ng) were assayed at different pH values as descibed in section II.9.a. 1 FU = .34 pmoles AMC released from the synthetic substrate, AAF-AMC. Standard deviations are calculated for n=3.

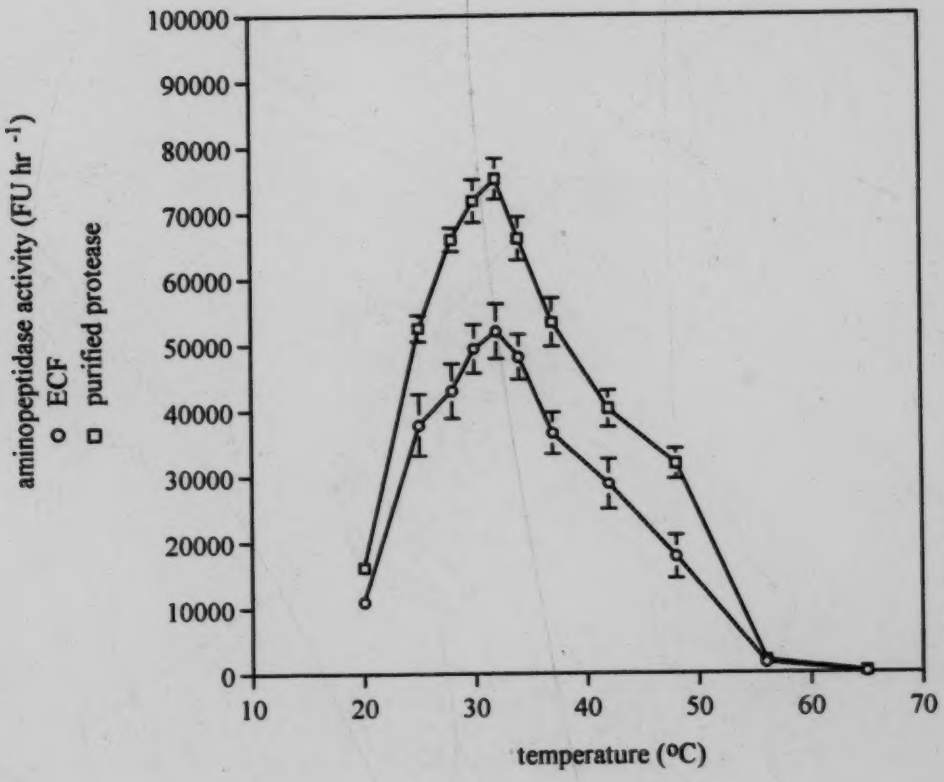


FIG. III-14. Effect of temperature on aminopeptidase activity in extracellular filtrate (ECF; 0.1 mg) and purified protease (5.0 ng). Assays were performed as described in section II.4. 1 FU = 0.34 pmoles AMC released from the synthetic suubstrate AAF- α-methylcoumarin (AAF-AMC). Standard deviations are calculated for n=3.

TABLE III-2. Effects of proteolytic inhibitors on aminopeptidase activity

inhibitor[b]	concentration (mM)	inhibition of aminopeptidase activity (%)[a]	
		ECF[c]	purified protease
amastatin	0.01	96.1 ± 1.1	98.9 ± 0.2
bestatin	0.01	94.2 ± 1.1	99.0 ± 0.2
3,4-DCI	0.10	2.4 ± 9.9	3.7 ± 4.8
E-64	0.01	3.0 ± 9.9	2.1 ± 1.4
EDTA	10.0	99.0 ± 0.2	98.8 ± 0.3
leupeptin	0.10	1.7 ± 0.6	1.2 ± 4.3
pepstatin	0.001	2.1 ± 3.0	0.3 ± 2.5
1,10-phenanthroline	1.0	100.0 ± 0.0	99.8 ± 0.3
PMSF	1.0	5.4 ± 6.4	5.5 ± 1.1
Zincov™[d]	0.1	94.6 ± 1.4	98.2 ± 0.1

[a] The effects of proteolytic inhibitors on enzyme activity in concentrated ECF and purified enzyme were measured. Results are presented as the % inhibition of aminopeptidase activity and standard deviations are reported for n=3.

[b] Descriptions of the effective inhibitors are provided in Chapter IV. Discussion.

[c] ECF = extracellular filtrate concentrate.

[d] ™ Calbiochem.

activity was not inhibited by the serine protease inhibitors, 3,4-DCI, PMSF and leupeptin. It was not inhibited by cysteine protease inhibitors, E-64 and leupeptin. Finally, there was no inhibition by pepstatin, an aspartic protease inhibitor (Table III-2).

III.4.d. Effect of metal ions on restoration of aminopeptidase activity

Metal ions were examined to determine their effects on activity of ECF and purified aminopeptidase previously treated with EDTA. During the design of the purification procedure, metal ions were assessed for their ability to restore aminopeptidase activity of SDS-PAGE denatured purified protease. Metal ions included during dialysis were $ZnCl_2$, $CaCl_2$, $MgCl_2$, $CoCl_2$, $MnCl_2$ or $FeCl_3$. $ZnCl_2$ restored activity best, $CaCl_2$ restored 30 % of the activity restored by $ZnCl_2$, and all of the other ions did not restore activity in that experiment. To examine restoration of activity after treatment with EDTA, $ZnCl_2$, $CaCl_2$ and $MgCl_2$ were examined. Addition of 10 mM EDTA eliminated aminopeptidase activity completely in both ECF and purified protease (Table III-3).

In the metal ion free ECF, $ZnCl_2$ restored maximum activity at 25 μM and activity decreased at concentrations $\geq$ .05 mM (Table III-3). $MgCl_2$ was only able to restore 11.8 % of activity at 10 mM. $CaCl_2$ also restored activity with a maximum of 80 % at 0.5 mM and activity decreased at $\geq$ 1 mM. In the metal ion free purified protease, $ZnCl_2$ restored activity most effectively, showing almost 100 % activity at 50 μM. Activity decreased at concentrations $\geq$ 2.0 mM (Table III-3). $MgCl_2$ only restored 30.0 % of activity at 10 mM. $CaCl_2$ also restored more than 88 % of activity at 100 μM and activity decreased at $\geq$ 2 mM. In the assays, there was visible

Table III-3. Effects of metal ions on restoration of aminopeptidase activity[a]

	ECF + 10 mM EDTA (% activity)			purified protease + 10 mM EDTA (% activity)		
ion conc (mM)	$ZnCl_2$	$MgCl_2$	$CaCl_2$	$ZnCl_2$	$MgCl_2$	$CaCl_2$
0.0	0.6 ± 0.6	0.9 ± 1.0	0.6 ± 0.6	0.2 ± 0.2	0.1 ± 0.2	0.4 ± 0.3
0.005	3.0 ± 2.6	1.1 ± 1.2	0.6 ± 0.5	6.1 ± 0.8	0.3 ± 0.2	0.6 ± 0.6
0.010	11.1 ± 5.2	0.7 ± 0.6	0.9 ± 0.8	48.9 ± 3.0	0.1 ± 0.1	6.7 ± 0.9
0.025	84.5 ± 7.9	0.7 ± 0.7	4.9 ± 2.0	76.2 ± 2.5	0.2 ± 0.2	20.9 ± 1.4
0.050	68.6 ± 7.2	0.8 ± 0.7	15.6 ± 6.5	97.9 ± 0.9	3.0 ± 1.2	73.1 ±2.8
0.10	62.6 ± 4.0	0.9 ± 0.8	30.9 ± 4.4	96.5 ± 1.0	6.1 ± 0.6	88.4 ± 2.2
0.25	61.1 ± 3.5	3.1 ± 2.7	61.8 ± 6.3	98.1 ± 0.7	6.2 ± 0.6	89.2 ± 1.8
0.50	47.5 ± 8.6	3.7 ± 2.7	79.4 ± 5.5	100.0 ± 2.1	6.7 ± 2.4	89.8 ± 1.9
1.0	27.1 ± 8.8	5.0 ± 3.0	78.7 ± 7.0	89.4 ± 1.3	16.3 ± 1.3	91.1 ±1.7
2.0	8.0 ± 4.0[b]	5.7 ± 3.9	69.8 ± 5.2	68.0 ± 2.6[b]	22.6 ± 1.8	77.5 ± 1.8
5.0	0.5 ± 0.5[b]	11.5 ± 4.8	26.3 ± 6.4[b]	0.37 ± 0.3[b]	28.5 ± 2.3	38.2 ± 2.7[b]
10.0	1.2 ± 1.3[b]	11.8 ± 5.4	5.8 ± 4.0[b]	0.25 ± 0.2[b]	30.0 ± 2.2	5.5 ± 0.8[b]

[a] The effects of metal ions on restoration of aminopeptidase activity were determined after treatment with 10 mM EDTA. There were 92.0 and 96.8 % recovery of enzyme activity after precipitation from ECF and purified protease, respectively. Results are presented as the relative percent of remaining aminopeptidase activity. Standard deviations are calculated for n=3.

[b] Precipitate was visible in wells after the 1 hour assay was complete.

precipitate in the $ZnCl_2$ containing wells at concentrations of 2.0, 5.0 and 10.0 mM and $CaCl_2$ containing wells at concentrations of 5.0 and 10.0 mM. Amino acid analysis of the precipitate revealed the presence of aminopeptidase in the precipitate.

III.4.e. Substrate specificity of the aminopeptidase

Aminopeptidase activity in ECF and purified protease was assayed against AMC-containing protease substrates with different amino acids at the P1 position (Table III-4). Activity was highest against the synthetic amino acid substrates with arg, phe, leu and ala at the P1 position. High activity against ala and phe was also demonstrated by its ability to cleave the synthetic oligopeptide substrate, AAF-AMC. Activity was very low against substrates with gly, ile, pro, ser, or val at the P1 position. There was no detectable activity in either ECF or purified protease against the substrate with glu at the P1 position. There was very low activity against N-terminally blocked substrates in ECF and no detectable activity against these substrates by the purified aminopeptidase. Activity profiles were similar for both ECF and purified protease.

Km, Vmax and turnover rates were determined for the substrates; R-AMC, L-AMC and P-AMC. FIG. III-15 is a direct linear plot used for the determination of Km and Vmax for aminopeptidase activity against the synthetic substrate, R-AMC. Similar plots were prepared for L-AMC and P-AMC (data not shown). Km values were 4.7, 19.2, and 36.2 μM, respectively. Vmax values were 317, 258 and 9.9 μmoles min^{-1} mg^{-1}, respectively. Turnover rates were 491, 400 and 15 s^{-1}, respectively (Table III-5).

TABLE III-4. Activity of ECF and Purified Aminopeptidase against AMC-releasing substrates

substrate[b,c]	Activity of ECF and purified protease against substrate (FU hr^{-1})[a]	
	ECF[d] (per μg)	purified protease (per ng)
AAF-AMC	106 ± 9.0	3,400 ± 200
N-succ-AAF-AMC	2.0 ± 0.3	3 ± 5
N-succ-GPLGP-AMC	11.8 ± 0.9	20 ± 20
A-AMC	190 ± 20	5,000 ± 100
R-AMC	520 ± 20	13,000 ± 1,000
E-AMC	0.4 ± 0.4	17 ± 20
G-AMC	6.0 ± 0.7	140 ± 10
I-AMC	8.2 ± 0.7	170 ± 10
L-AMC	210 ± 20	5,300 ± 200
F-AMC	280 ± 30	6,800 ± 3,000
P-AMC	10.4 ± 0.5	136 ± 2
S-AMC	8.3 ± 0.5	131 ± 9
V-AMC	1.8 ± 0.4	49 ± 5

[a] Fluorescence of enzyme/substrate assay mixture was read every 5 min for 1 hour. 1 FU = 0.34 pmoles AMC released from the synthetic substrate. Standard deviations are calculated for n = 3.

[b] Amino acids are represented by the one-letter code; N-succ- denotes an N-succinyl blocking group on the N-terminus of the substrate; AMC stands for α-methylcoumarin, the fluorescent leaving group.

[c] Substrates were all used at a concentration of 2.15×10^{-5} M.

[d] ECF = extrcallular filtrate concentrate.

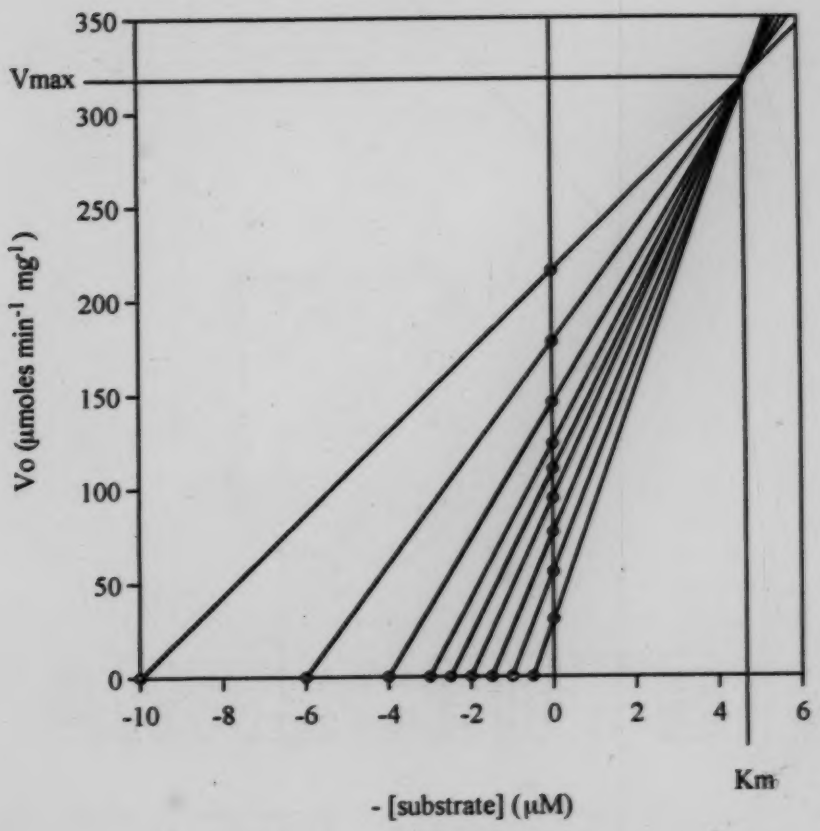


FIG. III-15. A direct linear plot for the determination of Km, the Michaelis-Menten constant, and Vmax, the maximum reaction rate, for the purified aminnopeptidase cleavage of the synthetic substrate, R-AMC (arginine-α-methylcoumarin). Vo = initial velocity.

TABLE III-5. Summary of Km, Vmax and turnover rate for purified aminopeptidase against R-AMC, L-AMC and P-AMC[a]

substrate[b]	Km (μM)[c]	Vmax (μmoles min^{-1} mg^{-1})[c]	turnover rate[c] (s^{-1})
R-AMC	4.7 ± .1	317 ± 20	491 ± 8
L-AMC	19.2 ± .2	258 ± 10	400 ± 9
P-AMC	36.2 ± .4	9.9 ± 1	15 ± 1

[a] Initial velocity was measured at substrate concentrations ranging from 0.5 to 40 μM with the substrate remaining in excess by at least 10-fold of the maximum fluorescence obtained.

[b] Amino acids are represented by the one-letter code; AMC stands for α-methylcoumarin, the fluorescent leaving group.

[c] Standard deviations are calculated for n=3.

III.5. Amino-terminal sequencing of the purified aminopeptidase

The N-terminal sequence of the purified protein was determined by Edman degradation and compared to other sequences. The first 34 amino acids from the N-terminus of the purified protease were 1-A-D-P-N-L-T-R-D-Q-A-V-E-R-A-A-L-I-T-V-D-S-Y-Q-I-I-L-D-V-T-D-G-N-G-A-34 (FIG III-16). A search for homologous sequences in Genbank was performed for both protein sequences and translated DNA sequences using the BLAST algorithm (Altschul *et al.*, 1990). A predicted aminopeptidase from *M. tuberculosis* virulent strain H37Rv, the *pepD* gene product, was found which was highly homologous to the N-terminal amino acids determined for our purified enzyme (Genbank accession no. AL021246; Phillip *et al.*, 1996; Cole *et al.*, 1998). The predicted 861 amino acid gene product has an N-terminal met, which is missing from our sequence, and a leu residue at position 3, which is an asp residue in our sequence (94 % similar residues). Another protein with homology is the *Streptomyces lividans* 66 *pepN* gene product that has 65 % identity and 88 % similar residues between amino acids 4 – 29 from the *M. tuberculosis* H37Ra sequence and amino acids 5 – 30 in the *S. lividans* translated protein sequence (Butler *et al.*, 1994).

III.6. Amount of SDS associated with the purified aminopeptidase

An assay to determine the amount of SDS associated with the purified protease was performed to see if the amount of SDS remaining associated with the protease was sufficient to effect its activity. A standard curve showing amount of SDS versus absorbance in this assay is shown in FIG III-17. The purified aminopeptidase had an A553 of 0.736 and 0.035, before and after dialysis,

H37Ra 1- A D P N L T R D Q A V E R A A L I -17
H37Rv pepD 1- M A L P N L T R D Q A V E R A A L I -18
S. lividans 66 pepN 5- N L T R E E A R Q R A T L L -18
H37Ra 18- T V D S Y Q I I L D V T D G N G A -34
H37Rv pepD 19- T V D S Y Q I I L D V T D G N G A -35
S. lividans 66 pepN 19- T V D S Y E I D L D L T -30

FIG. III-16. N-terminal amino acid sequence of purified aminopeptidase from strain H37Ra compared to the N-terminal sequence of the predicted aminopeptidase from strain H37Rv deduced from its DNA sequence and part of the N-terminal sequence from the *Streptomyces lividans pepN* gene product. Amino acids are represented by their one-letter code. Identical amino acids are indicated by a line and conservative amino acid changes are indicated by an asterisk.

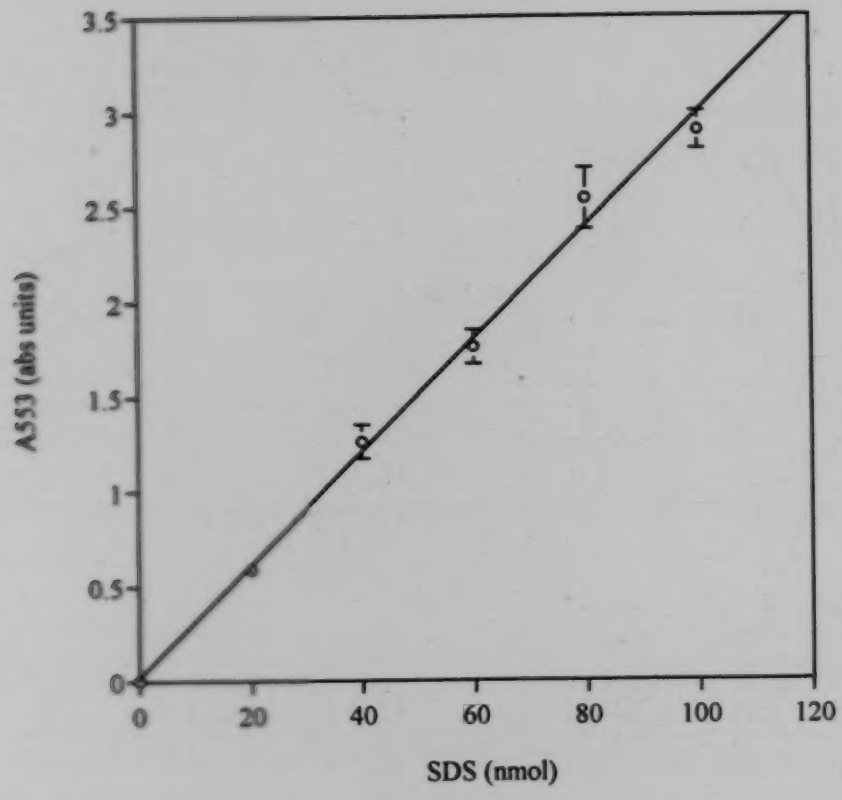


FIG. III-17. Standard curve to determine the amount of SDS remaining associated with the purified aminopeptidase. The absorbance at 553 nm (A553) of the electroeluted protease was 0.736 and the diluted and dialyzed protease was 0.035 refelecting SDS amounts of 24 and 0.6 nmoles, respectively.

respectively. This corresponds to 24 and 0.6 nmoles SDS or .007 % and .00016 %, respectively. Addition of SDS to purified protease showed that a concentration of 0.1 % was required to inhibit activity completely and even 0.05 % only inhibited 50 % of activity of the purified protease. When SDS was added to purified aminopeptidase to concentrations of .007 and .00016 %, 88 and 97 % of activity was retained, respectively.

III.7. Reaction between tuberculosis-positive patient sera and sonicate, ECF or purified protease

Son, ECF and purified protease were examined by Western immunoblotting to determine their reactivity with sera from tuberculosis-positive patients. FIG. III-18a is the silver-stained SDS-polyacrylamide gel of son, ECF and purified protease. FIG. III-18b and FIG. III-18c are Western blots of son, ECF and purified protease detected with sera from two different tuberculosis-positive patients. There is reaction between both sonicate and ECF and both patient sera at several protein bands ranging in molecular weight from approximately 66 kDa to the running front of the gel. There is no reaction between the patient sera and the purified aminopeptidase.

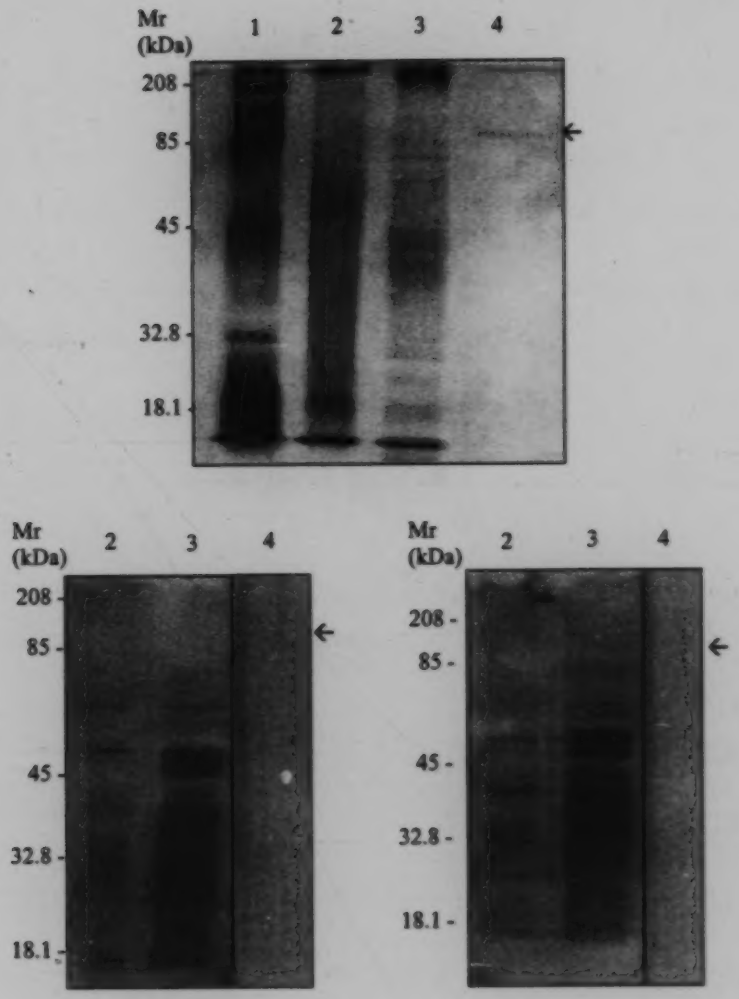


FIG. III-18. Silver stained SDS-PAGE analysis (a) and western immunoblots detected with tuberculosis-positive patient sera (b and c) of son, ECF and purified protease. Immunoblot detection was performed with alkaline phosphatase-labeled goat anti-human antibodies. Kaleidoscope molecular weight markers: myosin (Mr 208 kDa), bovine serum albumin (Mr 85 kDa), carbonic anhydrase (Mr 45 kDa), soybean trypsin inhibitor (Mr 32.8 kDa) and lysozyme (Mr 18.1 kDa; lane 1). Son (lane 2), ECF (lane 3) and purified protease (lane 4). Mr = molecular weight; kDa = kilodaltons.

Chapter IV. Discussion

IV.1. Purification of the *M. tuberculosis* aminopeptidase

IV.1.a. Selection of material used for purification

Mycobacterium tuberculosis strain H37Ra (an avirulent strain) was used for the production of starting material to minimize the health risks to laboratory personelle.

The starting material chosen for purification was 4 week ECF because culture growth, aminopeptidase activity and collagenase activity were still increasing up to 4 weeks (FIG. III-1a and FIG A1-1a). ECF was chosen as starting material over sonicate despite higher aminopeptidase and collagenase activity in sonicate. This was to reduce contamination from intracellular components, which would be found exclusively, or in larger amounts, in whole cell sonicates. Aminopeptidase (but not collagenase) in ECF increases more dramatically at weeks 5 and 6 but was not used for isolation starting material. Culture growth did not increase to the same degree as the increase in aminopeptidase activity and this suggests that the aminopeptidase and other intracellular enzymes may be in the mixture due to cell lysis.

IV.1.b. Evaluation of purification achieved

The *M. tuberculosis* H37Ra aminopeptidase activity has been purified from 4 week ECF to apparent homogeneity as shown by silver staining of an SDS-polyacrylamide gel (FIG. III-9) and by the presence of only one unblocked protein during N-terminal sequencing.

IV.2. Characterization of the aminopeptidase protein

IV.2.a. The molecular weight of the purified aminopeptidase

The native protein is 80 $\pm$ 5 kilodaltons by SEC, which is similar to the molecular weight of approximately 75 kilodaltons determined by Gleisner and Ramthun (1981). The denatured protein is 93 kilodaltons by SDS-PAGE. The

predicted molecular weight of the 861 amino acid translated gene product from *M. tuberculosis* H37Rv is 94,226 daltons. The DNA sequence does not predict the molecular weight of native proteins because the folded structure affects the observed molecular weight of proteins. After introduction into the macrophage, a nutrient limiting environment (Schlesinger, 1996), *M. avium* up-regulates several proteins. One of them is 95 kDa (Bermudez and Petrofsky, 1997) and may correspond to the aminopeptidase described here.

IV.2.b. The pI of the purified aminopeptidase

The pI of the protein is 4.1 ± .1 by chromatofocusing (FIG. III-12). The pI of the native H37Rv protein, calculated from its predicted amino acid composition is 4.5 ± .5 (Patrickios and Yamasaki, 1995). The charge ratio was used and is reported to be accurate to within 1 pH unit for the proteins surveyed and 0.5 pH units for proteins surveyed with pIs ranging from 3 – 5 (Patrickios and Yamasaki, 1995). The amino acid charge ratio for the predicted aminopeptidase from H37Rv is consistent with that found for H37Ra by amino acid analysis.

IV.3. Characterization of the aminopeptidase enzyme activity

IV.3.a. Aminopeptidase pH and temperature optima

Proteins are charged molecules. The charge of a protein at a certain pH is determined by the charge state of the ionizable groups on the protein. Ionizable groups include the amino terminus (pKa = 9.55) and the carboxy terminus (pKa = 2.18) as well as the amino acid side chains (Lehninger, 1982b). The pKa's for the R-groups of D, E, H, C, Y, K, and R are 3.86, 4.25, 6.0, 8.33, 10.07, 10.53 and 12.48, respectively (Lehninger, 1982b). The local environment in the folded protein also effects the pKa of an amino acid side chain. At any pH, proteins carry a net charge which affects the overall conformation of the protein (Lehninger, 1982b). Similarly, pH affects protease substrates with ionizeable amino acid side chains. At an

optimal pH for activity, the charge on the enzyme will convey the correct conformation for optimal activity.

The pH optimum of the purified aminopeptidase is 7.0 consistent with that of a neutral protease (FIG. III-13). The pH range for activity was 5.5 - 8.5 which may be optimal for a bacterium which commonly inhabits a slightly acidified phagosome (Crowle *et al.*, 1991). The normal pH of ECF from *M. tuberculosis* is 7.4 and pH of tuberculous pleural fluid is $\leq$ 7.3 (Henry, 1984). The mycobacterial aminopeptidase can exhibit activity in all of these environments. Consistent with our results, bacterial aminopeptidases tend to have broad pH ranges, from 6-9 (Gonzales and Robert-Baudouy, 1996).

Below optimal temperature for activity, enzymes and substrates have less kinetic energy available and fewer reactions occur (Tipton, 1992). Above optimal temperature for activity, enzymes thermally denature or partially unfold reducing their activity (Tsou, 1995).

The temperature optimum for the *M. tuberculosis* aminopeptidase is 32°C and activity is present at a temperature as high as 42°C (FIG. III-14). This may be ideal for an enzyme which exists within alveolar macrophages where temperatures are consistently below core temperature (37°C; Ganong, 1997) and may be as low as 32°C (Crowle *et al.*, 1991). Its stability and activity at higher temperatures are important because temperatures are as high as 42°C in a human host with fever (Clemens *et al.*, 1995). Consistent with this result, bacterial aminopeptidases usually have temperature optima similar to the growth conditions encountered by the bacterium (Gonzales and Robert-Baudouy, 1996). Enzyme activity, though it may not be optimal, would still be present in a wide range of environments in the human host including, most importantly, after internalization into macrophages.

IV.3.b. Inhibition profile of the aminopeptidase

Protease inhibitors used in this study and a brief description of their activity are shown in Table IV-1. The aminopeptidase is inhibited by amastatin and bestatin, EDTA and 1,10-phenanthroline as well as by Zincov™ (Table III-2). Amastatin and bestatin are transition state oligopeptide and dipeptide analogs, respectively (Benyon and Salvesen, 1993; Taylor, 1995). They inhibit aminopeptidase activity by competitively binding at the active site (Benyon and Salvesen, 1993) but are not cleaved by the enzyme because there is an extra carbon atom between the α-carbon and the carboxyl carbon of the substrate backbone. The carboxyl carbon is the normal site of nucleophilic attack in the formation of the protease tetrahedral intermediate and is not in the correct position for attack by the enzyme due to the extra carbon atom (Taylor, 1995).

Inhibition by EDTA and 1,10-phenanthroline support a requirement for a metal cofactor for activity, as both chelate metal ions (Benyon and Salvesen, 1993). 1,10-phenanthroline has a higher specificity for Zn^{2+} than for Ca^{2+} with affinity constants of 2.5×10^6 M^{-1} and 3.2 M^{-1}, respectively (Salvesen and Nagase, 1993). When used at a concentration of 1 mM, 1,10-phenanthroline chelates active site zinc ions but not bound calcium, which can be present in all classes of proteases to stabilize conformation (Salvesen and Nagase, 1993). Thus, 1,10-phenanthroline is specific for zinc metallo-proteases, unlike EDTA, which inhibits metallo-proteases as well as metal-requiring proteases. Zincov™ inhibition further supports that the cofactor required may be zinc since it inhibits members of the zinc-requiring metalloprotease family. It is a hydroxamic acid tripeptide analogue, which competitively binds at the active site (Hudgin *et al.*, 1981). The substrate analogue is not cleaved because it binds in a sterically inappropriate conformation for cleavage. There is an extra carboxyl carbon on the N-terminal side of the substrate α-carbon, which, together with

Table IV-1. Protease inhibitors[a]

inhibitor	class	description[b]
amastatin	metallo-	reversible, competitive inhibitor of aminopeptidases, apA , LAP
bestatin	metallo-	reversible, competitive inhibitor of aminopeptidases, apB, LAP, apN
3,4-DCI	serine	irreversible inhibitor of serine proteases; acylates active site serine
E-64	cysteine	irreversible inhibitor of cysteine proteases; active site titrant
EDTA	metallo- or metal-activated	reversible inhibition by metal chelation
leupeptin	serine and cysteine	reversible inhibitor of trypsin-like serine proteases and some cysteine proteases
pepstatin	aspartic	reversible inhibitor of cathepsin D , pepsin, and renin
1,10-phenanthroline	metallo-	reversible inhibition by metal chelation; high affinity for Zn^{2+}, low for Ca^{2+}
PMSF	serine	irreversible inhibitor; sulfonylation of active site serine
TLCK	serine	irreversibly inhibits trypsin-like serine proteases; no effect on chymotrypsin
TPCK	serine	irreversibly inhibits chymotrypsin-like serine proteases; no effect on trypsin
Zincov™.[c]	metallo-	reversible, competitive inhibitor of zinc-containing metalloproteases

[a] Reproduced from Benyon and Salvesen (1993).

[b] Thorough descriptions of effective inhibitors are in section IV.3.b.

[c] ™ Calbiochem.

the carboxyl carbon of the backbone, interact with the catalytic zinc ion effecting its orientation (Hudgin *et al.*, 1981). Serine, cysteine and aspartic protease inhibitors assayed were ineffective. In total, this indicates that the enzyme is a zinc-requiring aminopeptidase. Two-thirds of bacterial aminopeptidases are metalloproteases and they most frequently use Zn^{2+} as their cofactor (Gonzales and Robert-Baudouy, 1996).

IV.3.c. Restoration of aminopeptidase activity by metal cations

The restoration of activity by 50 μM $ZnCl_2$ after chelation by EDTA is consistent with a zinc-requiring enzyme; however, calcium also restores activity effectively (Table III-3).

Though bacterial aminopeptidases, most commonly use Zn^{2+} as their metal cofactor, some aminopeptidases can use other ions as well (Gonzales and Robert-Baudouy, 1996). The *Streptomyces griseus* leucine aminopeptidase has Zn^{2+} at its active site but this enzyme can be replaced with Ca^{2+} changing the catalytic activity and specificity of the enzyme (Ben-Meier *et al.*, 1993). It has been proposed that this may provide a mechanism of regulation of protease activity *in vivo* (Ajabnoor and Wagner, 1979).

Inhibition by μM concentrations of Zn^{2+} and other heavy metal cations has been reported previously (Salvesen and Nagase, 1993). To investigate the cause of a decrease in restoration of activity by metal cations, we performed an amino acid analysis on the precipitate formed in assay wells. There was aminopeptidase present in the wells. The efficacy of ions at salting in and salting out proteins is shown below and has been reproduced from Harris and Angal (1989).

Increasing salting-out effect

PO_4^{2-}, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-

NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}

Increasing salting-in effect

We use a phosphate buffer system for maintaining the pH of our assay mixture and it is the strongest salting out anion listed. Perhaps this is leading to the salting out of our protein from solution during the 1 hour assay.

Decrease in reactivation of aminopeptidase activity occurs at 1 mM $ZnCl_2$ for purified aminopeptidase but at 0.05 mM $ZnCl_2$ for crude ECF (Table III-3). There are two differences between the enzyme in each of these environments. The first difference is the presence of other proteins in ECF. These may effect the activity of aminopeptidase in ECF by co-precipitating it at lower concentrations of $ZnCl_2$ thereby reducing activity or there may be other zinc metalloproteases activated in ECF which degrade our protease reducing activity. Secondly, and perhaps more importantly, there is a much lower amount of aminopeptidase present in ECF than in the purified form. Larsen and Auld (1991) have described inhibition of a carboxypeptidase by excess zinc cations as being due to the formation of a bridge between zinc monohydroxide and an amino acid side chain at the active site of the enzyme. In ECF, there is a much lower proportion of zinc-requiring enzymes than in purified protease. Perhaps the competitive inhibition by zinc can occur at lower concentrations because there are proportionally fewer sites available in ECF versus purified enzyme for binding extra zinc ions.

To definitively answer the question of which ion is required at the active site of a metalloprotease, Leopold and Fricke (1997) have used size exclusion chromatography by high-pressure liquid chromatography coupled to plasma mass

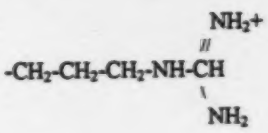
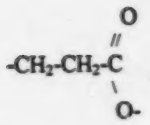
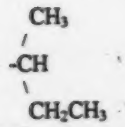
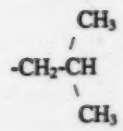
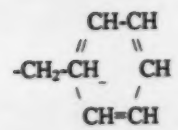
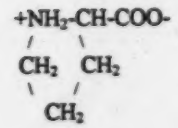
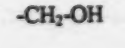
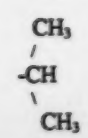
spectroscopy to identify and quantitate the metal ion requirement of some metalloproteases.

IV.3.d. Substrate specificity of the aminopeptidase

Table IV-2 shows the structure of the amino acid side chains for the amino acids used to determine the substrate specificity of the *M. tuberculosis* aminopeptidase. The purified aminopeptidase has highest activity against R in the P1 position. It is active against the amino acid substrates with N-terminal F, L or A (Table III-4). This suggests that it has a preference for hydrophobic amino acid side chains. There was no or extremely low activity against amino acid substrates with G, I, P, S, V or E at the P1 position (Table III-4.). To ensure that the glu substrate was accessible to the enzyme, we used SV8 protease (Calbiochem-Novabiochem Corp., La Jolla, California, USA) against the substrate, which specifically cleaves on the C-terminal side of a glu residue. It was able to digest the synthetic substrate, E-AMC. Finally, there was no activity against N-terminally blocked oligopeptide substrates confirming that the activity observed is that of an aminopeptidase (Table III-4).

Aminopeptidase substrate specificity is determined by two interactions between the enzyme and substrate, which orient the substrate correctly so that cleavage by the enzyme active site can occur. The first interaction is with the substrate's charged N-terminus that may be provided by negatively charged amino acid side chains from the enzyme as was shown for the proline iminopeptidase from *Xanthomonas campestris* (Medrano *et al.*, 1998). Alternatively, the charged N-terminus may be coordinated to an active site zinc ion as was shown for bovine lens aminopeptidase (Strater and Lipscomb, 1995). The second interaction is with the amino acid side chain at the P1 position and the aminopeptidase binding pocket. The binding pocket for our aminopeptidase is likely hydrophobic because the enzyme cleaves after hydrophobic

Table IV-2. Amino acid R-groups[a]

amino acid	R-group
alanine	
arginine	
glutamic acid	
glycine	
isoleucine	
leucine	
phenylalanine	
proline[b]	
serine	
valine	

[a] R-groups are reproduced from Lehninger (1982a).

[b] The diagram of proline shows the zwitterionic form of the amino acid instead of the R-group because the delta carbon of the R-group is covalently bonded to the α-amino group of the amino acid.

amino acids well. In support of this conclusion, the crystallographic structure of bestatin bound at the active site of bovine lens leucine aminopeptidase showed the F-like side chain of bestatin bound in a hydrophobic pocket made of 5 amino acid side chains from the protease (Taylor, 1993). Similarly, the crystal structure of proline iminopeptidase from *Xanthomonas campestris* revealed five hydrophobic amino acid side chains from the protease forming a small hydrophobic binding pocket for the N-terminal proline (Madrena *et al.*, 1998). High activity against R-AMC and no activity against E-AMC suggest that there may be a negative charge near or within the hydrophobic binding pocket. E-AMC may also be excluded from the binding pocket by the nearby negative charge hypothesized to interact with the N-terminal charged group.

It is interesting that I-AMC and V-AMC are not cleaved well by the enzyme. They are both hydrophobic amino acids but differ from the others assayed in that they are β-branched (Table IV-2). This means the side chain becomes larger near the α-carbon of the polypeptide backbone. A binding pocket could exclude β-branched amino acid side chains by being narrow at the position that would bind the β-carbon atom. Alternatively, the slow rate of reaction observed may not reflect the synthetic substrate's inability to bind in the hydrophobic binding pocket, but may reflect the inability of the enzyme to cleave the N-terminal amino acid due to the orientation of the substrate within the binding pocket.

Gonzales and Robert-Baudouy (1996) summarized and classified the known bacterial aminopeptidases. Our aminopeptidase is similar to the PepN family which are typically zinc-requiring metalloproteases that function at neutral pH with molecular weights ranging from 87 to 99 kDa. The substrate specificity for this family is for A, L, K or R at the P1 position (Gonzales and Robert-Baudouy, 1996). These proteases typically have no activity if an acidic residue (D, E or pyroglutamic acid) is in the P1

position. Ours has no activity against E in the P1 position. Members of this family usually have no activity if proline is in the P1 or P1' position (Gonzales and Robert-Baudouy, 1996). Ours shows extremely low activity against P-AMC.

The *M. tuberculosis* aminopeptidase demonstrates Michaelis-Menten kinetics. Km, Vmax and turnover rate were determined for R-AMC, L-AMC and P-AMC (Table III-5). Km is the substrate concentration at which half of the enzyme active sites are full. Further, if the rate of dissociation of the enzyme-substrate complex is high in comparison to the reaction rate, Km also reflects binding strength (Stryer, 1995a). Because Km is four-fold higher for L-AMC (19.2 μM) versus R-AMC (4.7 μM) and the Vmax is 81 % of that observed for R-AMC, the aminopeptidase must have higher binding affinity for R-AMC. The Km for P-AMC (36.2 μM) is 7.7 fold higher than that of R-AMC but the P-AMC Vmax is only 3 % of that observed for R-AMC. This could be explained if the binding of P-AMC and R-AMC was comparable but cleavage was very slow. Km values for aminopeptidases range from 3 μM for the *Lactobacillus helveticus* broad specificity aminopeptidase (Sasaki *et al.*, 1996) to 1 mM for bovine lens aminopeptidase (Taylor, 1993) so our aminopeptidase shows a high relative binding affinity compared to others.

Vmax represents the maximum rate of reaction and turnover rate is the number of molecules of product produced per molecule of enzyme when all binding sites are filled in a defined amount of time (Stryer, 1995a). Vmax is 317, 258 and 9.9 μmoles min-1 mg-1 for R-AMC, L-AMC and P-AMC, respectively. The turnover rate is 491, 400 and 15 s^{-1} for R-AMC, L-AMC and P-AMC, respectively. Vmax for aminopeptidases range from 2.5 μmoles for the *Lactobacillus helveticus* aminopeptidase (Sasaki *et al.*, 1996) to 350 mmoles min^{-1} mg^{-1} for the *Lactobacillus helveticus* iminopeptidase (Varmanen *et al.*, 1996). Vmax and turnover rate for R-AMC and L-AMC demonstrate that the aminopeptidase possesses an intermediate

rate of reaction against these substrates. P-AMC shows a much lower rate of reaction and is a less ideal substrate for this protease. Activity against P is low and not reported for most aminopeptidases from this family. The *S. lividans* aminopeptidase has highest activity against L-*p*-nitroanilide and activity against P-*p*-nitroanilide is 7 % of that seen for L-*p*-nitroanilide (Butler *et al.*, 1994). Typical proline aminopeptidases have turnover rates of 654 s^{-1} (Walter *et al.*, 1980). The turnover rate of P-AMC for our enzyme (15 s^{-1}) suggests that it may not be effective *in vivo* against peptides with N-terminal P. The H37Rv genome sequence predicts the presence of a separate proline iminopeptidase (Cole *et al.*, 1998).

IV.4. The *Mycobacterium tuberculosis* aminopeptidase sequence

IV.4.a. N-terminal sequence of the H37Ra aminopeptidase

The purified enzyme from *M. tuberculosis* H37Ra very closely matches the predicted enzyme sequenced from *M. tuberculosis* H37Rv, the *pepD* gene product, having identical amino acids at 33 of 35 sites at the N-terminus (FIG. III-16; Altschul *et al.*, 1990; Cole *et al.*, 1998). The N-terminal met residue predicted from the DNA sequence for H37Rv is not present in our purified protease and may be cleaved post-translationally in the H37Rv enzyme. Methionine aminopeptidases predicted from the gene sequence of H37Rv (Cole *et al.*, 1998) show homology to the *E. coli* methionine aminopeptidase which cleaves N-terminal met well if the residue at the P1' position is ala (Ben-Bassat *et al.*, 1987), as it is for the *M. tuberculosis* aminopeptidase predicted from H37Rv and our enzyme from H37Ra.

The second difference is a change from L to D at position 2 in our sequence. This change is not conservative at either the protein or the DNA level. A change in positions 1 and 2 of the DNA codon are required for this amino acid change. Our sequence was determined from three independent isolations using different freezer stocks for the growth of H37Ra each time. There may be microheterogeneity between

the proteases from different strains. Determination of the molecular weight of an aminopeptidase activity from *M. tuberculosis* by Gleisner and Ramthun (1981) revealed a molecular weight of 76 kDa for the native enzymes from strain H37Rv and a clinical isolate but a molecular weight of 75 kDa for the same enzymatic activity from strain H37Ra.

The N-terminal amino acid sequence from *M. tuberculosis* strain H37Ra has 65 % identity and 88 % homology to the N-terminus of the aminopeptidase N from *Streptomyces lividans* 66. This intracellular, neutral, zinc-requiring, broadly specific enzyme is the organism's predominant aminopeptidase activity. Its highest activity was against substrates with L, K or R at the P1 position. It showed 20 to 60 % of maximum activity against A, M, S, or F at the P1 position and no or low activity against P and G at the P1 position (Butler *et al.*, 1994). This is similar to the profile of our aminopeptidase with the exception of S, which had 20 % activity in their assay and only 2 % activity in ours. They did not establish the Vmax for the substrates assayed which would be required for an accurate comparison of relative substrate reaction rates.

M. tuberculosis H37Ra aminopeptidase amino acids 18 - 33 show homology with the human cell adhesion protein, cadherin, amino acids 1 - 16 with 56 % identity and 75 % similarity in the 16 amino acid stretch. Molecular mimicry of host proteins by tuberculous antigens has been implicated in autoimmune disorders (Chan *et al.*, 1995; Harris *et al.*, 1995) and our enzyme is a potential candidate for this mimicry.

There is no leader sequence present on the protein, therefore it is not actively secreted in *M. tuberculosis* by the general secretory pathway. It may be an extracellular protease excreted by an alternative mechanism or it may be an intracellular protein present in culture filtrates due to cell lysis. Gram-negative "*Serratia* protease like" metallo-proteases, which include the *Serratia* protease,

Pseudomonas alkaline protease and the *Erwinia* proteases, do not contain a leader peptide but are secreted by specific secretion proteins (Hase and Finkelstein, 1993). Specific secretion proteins have been identified for some bacterial toxins. An intact C-terminus is required for their secretion but the toxins show no obvious homology in this region. Homology does exist between the three toxin secretion pathway specific proteins and similar proteins have been predicted in *M. tuberculosis* from the genome sequence (Delepelaire and Wandersman, 1990; Cole *et al.*, 1998).

Regardless of cellular location, cytoplasmic or secreted, the aminopeptidase may play a role in nutrient acquisition or virulence. A secreted enzyme would have access to host proteins and secreted mycobacterial proteins which could be degraded for nutrient acquisition. Release of amino acids could also be used by the organism for ammonia production. This is also true of a cytoplasmic enzyme. Peptides could be imported into the *M. tuberculosis* cytoplasm and further degraded by an intracellular aminopeptidase for nutrient procurement and/or ammonia production. *M. tuberculosis* has been predicted to contain mechanisms for importing peptides on the basis of its genome sequence (Cole *et al.*, 1998). The urease enzyme, which is the predominant source of ammonia production, is located intracellularly (Clemens *et al.*, 1995) and there is a predicted ammonia export system found in the genome sequence as well (Cole *et al.*, 1998).

IV.4.b. Aminopeptidases described from the gene sequence of *M. tuberculosis* H37Rv

There are 9 aminopeptidase activities predicted from the genome sequence (Table IV-3). These include 6 cytoplasmic enzymes which are predicted to have highly specific activities (Cole *et al.*, 1998). There are three additional aminopeptidases predicted to have broad specificity. Aminopeptidase A/I, coded by the *pepB* gene, and aminopeptidase I, coded by the *pepC* gene, are homologous to

Table IV-3. Summary of *Mycobacterium tuberculosis* H37Rv proteases predicted from the genome sequence[a]

Genbank designation	gene designation	description
Rv3596c	*clpC*	ATP-dependent clp protease
Rv2461c[c]	*clpP*	ATP-dependent clp protease proteolytic subunit
Rv2460c[c]	*clpP2*	ATP-dependent clp protease proteolytic subunit
Rv2457c	*clpX*	ATP-dependent clp protease ATP-binding subunit, clpX
Rv2667[c]	*clpX'*	similar to clpC from *M. leprae* but shorter
Rv3419c	*gcp*	glycoprotease
Rv1223[c]	*htrA*	serine protease
Rv2861c[b]	*map*	methionine aminopeptidase
Rv0734[b]	*map'*	probable methionine aminopeptidase
Rv0319[b]	*pcp*	pyrrolidone-carboxylate peptidase
Rv0125[c]	*pepA*	probable serine protease
Rv2213[b]	*pepB*	aminopeptidase A/I
Rv0800[b]	*pepC*	aminopeptidase I
Rv2467[b]	*pepD*	probable aminopeptidase
Rv2089c[b]	*pepE*	cytoplasmic peptidase
Rv2535c[b]	*pepQ*	cytoplasmic peptidase
Rv2782c	*pepR*	protease/peptidase, M16 family (insulinase)
Rv0782	*ptrBa*	protease II, subunit
Rv0781	*ptrBb*	protease II, subunit
Rv0724	*sppA*	protease IV, signal peptide peptidase
Rv0198c	-	probable zinc metalloprotease
Rv0457c[c]	-	probable peptidase
Rv0840c[b,c]	-	probable proline iminopeptidase
Rv0983[c]	-	probable serine protease
Rv1977	-	probable zinc metallopeptidase
Rv3668c[c]	-	probable alkaline serine protease
Rv3671c[c]	-	probable serine protease
Rv3883c[c]	-	probable secreted protease
Rv3886c[c]	-	protease

[a] Reproduced from Cole *et al.* (1998).

[b] Indicates proteases which are possible aminopeptidases.

[c] Indicates proteases with predicted or possible serine active sites.

E. coli leucine aminopeptidase. The final aminopeptidase, encoded by the *pepD* gene, shares a high degree of N-terminal sequence homology with our purified aminopeptidase. This is the first demonstration of an identified protease activity associated with a specific gene product in *M. tuberculosis*. The fact that, even with losses inevitable during purification, we have recovered 25 % of original activity strongly suggests that it is a major contributor to aminopeptidase activity. Also, the similarity of the profile of activity in ECF against different substrates and the profile of our purified protease also argues that it is a major contributor to the activity found in four-week-old culture filtrates. DNA sequence does not predict expression of gene products; whether they are produced or not, whether they contribute large, small or trace amounts to the observed activity, or whether their expression is constitutive or induced. Perhaps *pepB* and *pepC* gene products are not produced, perhaps they are not present in the extracellular filtrate or are minor components contributing to the observed activity, or perhaps they are not induced under our growth conditions. Further, their substrate specificities may extend beyond the P1 position, making them ineffective against the synthetic substrates tested.

The predicted H37Rv *pepD* gene product has a zinc binding motif (HEXXH, amino acids 299-303) like that described for coordination of the zinc ligands in *Bacillus thermoproteolyticus* and other neutral, zinc-requiring proteases (Vallee and Auld, 1990). These two his residues along with a glu residue at position 322 are predicted to coordinate the zinc atom. Glu 300 is the predicted nucleophile for the reaction and tyr 214 may act as the active site proton donor (Garcia-Alverez *et al.*, 1991).

The predicted protein sequence from the H37Rv genome, has homology with six previously identified human aminopeptidases; human, microsomal, membrane alanyl, glutaminyl, placental and puromycin sensitive aminopeptidases. This provides a

mechanism for cross-reactivity between the H37Rv protein and human proteins, which is hypothesized to be a source of autoimmune disorders (Chan *et al.*, 1995; Harris *et al.*, 1995).

The predicted protein sequence also has short regions of homology with four protein sequences predicted by the presence of open reading frames in *M. tuberculosis* H37Rv with 36 – 46 % identity and 46 – 59 % homology in 26 – 39 amino acid stretches. These proteins have no predicted functions from their gene sequence.

There are also homologous regions with HIV type 1 envelope glycoprotein, gp41 (38 % identity and 62 % homology in a 26 amino acid sequence) and the *nef* gene product (36 % identity and 64 % homology in a 14 amino acid sequence; Altschul *et al.*, 1990).

IV.5. SDS associated with purified aminopeptidase

The amount of SDS present in the electroeluted samples before and after they were dialyzed was measured by the method of Waite and Wang (1976). The amount of SDS present in the dialyzed, active aminopeptidase was 0.035 nmoles or 0.00016 %. When this concentration of SDS was added to purified, active enzyme, 97 % of protease activity was retained. This suggests that the amount of SDS present in the purified sample is not sufficient to cause a significant decrease in enzyme activity. A number of methods have been used successfully to remove SDS from proteins even after SDS-PAGE with the restoration of activity (Dong *et al.*, 1997). Procedures that have been used include organic solvent precipitation, dialysis and buffer exchange by chromatography; reverse-phase high-pressure liquid chromatography, ion exchange chromatography or size exclusion chromatography. It is clear that denaturation by SDS is not an inevitably irreversible process (Dong *et al.*, 1997). A key factor in reconstitution of enzyme activity after SDS-PAGE is that samples can not be boiled in the presence of SDS and reducing agents, procedures that

are followed during the purification of our enzyme (Dong *et al.*, 1997). One theory of the mechanism for refolding of proteins after SDS-PAGE is that the SDS may have limited access to the hydrophobic core of the protein under these milder conditions preventing the complete unfolding of structural domains (Dong *et al.*, 1997).

IV.6. Reactivity of tuberculosis-positive patient sera with son, ECF and purified protease

Sera from two patients with tuberculosis reacted against son and ECF but not against the purified protease. This indicates that these two individuals did not have an antibody response to the aminopeptidase, detectable by our assay. Two patients provide a small sample size which may not accurately reflect a typical antibody response. Alternatively, the aminopeptidase may not be produced by *M. tuberculosis in vivo*, it may not be produced by the isolate(s) infecting the individuals, or it may be produced in too small an amount for the host to mount an antibody response to it. It may not be immunogenic because of structure or because shared epitopes with human proteins which could lead to a destructive auto-immune response. Finally, it may not be recognized by antibody if it is an intracellular *M. tuberculosis* antigen which is not accessible to the host immune response.

IV.7. Comparison to previously identified protease activities from *Mycobacterium tuberculosis*

IV.7.a. Aminopeptidase activity

Table IV-4 summarizes the properties of our aminopeptidase compared to those described previously. The properties of our aminopeptidase are similar to those described for an aminopeptidase from strains H37Ra, H37Rv and a clinical isolate (Gleisner and Ramthun, 1981). This protein digested synthetic substrates with an N-terminal K, L or A. Digestion of the synthetic amino acid substrates A-AMC and L-AMC demonstrates that our protein also cleaves N-terminal A or L. A molecular

Table IV-4. Comparison of our aminopeptidase with previously identified aminopeptidase activities from *M. tuberculosis*[a]

property	our aminopeptidase	Gleisner and Ramthun (1981)[c]	Wasem *et al.* (1991)[c]
strain	H37Ra	H37Rv, H37Ra, clinical isolate	*M. tuberculosis - bovis*
source	ECF or sonicate	whole cell lysates	whole cell lysates
Mr – native (kDa)	80 ± 5	75 – 76	ND
Mr – denatured (kDa)	93	ND	ND
pI (pH units)	4.1 ± .1	ND	ND
pH optimum (pH units)	7.0	7.0	ND
pH range (pH units)	5.0 – 8.5	5.5-8.0	ND
T optimum (°C)	32	ND	ND
T range (°C)	20 – 42	ND	ND
effective inhibitors	Amastatin, bestatin	ND	ND
	EDTA	EDTA	ND
	1,10-phenanthroline	1,10-phenanthroline	ND
	Zincov™[b]	ND	ND
substrate specificity	R, L, A, F	K, L, A	L

[a] Abbreviations: Mr, molecular weight; kDa, kilodaltons; T, temperature; inhibitors are described in Table IV-1. and in section IV.3.b.; amino acids are represented by their one-letter code.

[b] ™ Calbiochem.

[c] ND = not determined.

weight of approximately 75 kDa was determined by size exclusion chromatography for the native proteins which is similar to our finding of 80 ± 5 kDa. The pH optimum reported was 7.0; activity was present between a pH of 5.0 and 8.0; the enzyme was inhibited by metal chelators EDTA and 1,10-phenanthroline (Gleisner and Ramthun, 1981) similar to ours. This evidence strongly suggests that the activities described are due to the same enzyme.

The leucine aminopeptidase activity detected by multilocus enzyme electrophoresis could also be due to our enzyme. Characterization of the leucine aminopeptidase has not been done (Wasem *et al.*, 1991) making comparison difficult.

IV.7.b. Protease activity

Rowland *et al.* (1997) identified a protease in stationary phase culture filtrates of *M. tuberculosis* that cleaved elastin and casein. The variety of classes of inhibitors that were partially effective against this enzyme activity strongly suggests that it is due to more than one protease and may include this aminopeptidase. The degradation of elastin by mycobacterial proteases would result in tissue destruction and may increase susceptibility to respiratory infection and chronic lung disease (Rowland *et al.*, 1997).

IV.8. Potential role of a mycobacterial aminopeptidase

The role of an aminopeptidase in *M. tuberculosis* may be two fold. Bacterial aminopeptidases have been implicated as important in functions such as protein turnover. In doing so, they may also eliminate abnormal proteins and cleaved signal peptides producing amino acids (Gonzales and Robert-Baudouy, 1996). During amino acids catabolism, amino-terminal groups are removed and produce ammonia while the carbon skeleton produces intermediates of the citric acid cycle (Stryer, 1995b). Amino acid catabolism therefore produces nitrogen, carbon and energy sources for the bacterium.

IV.8.a. Role in mycobacterial growth

The ubiquitous presence of aminopeptidases in both Gram-positive and Gram-negative bacteria suggests that they may be critical to survival by providing housekeeping functions (Hase and Finkelstein, 1993). A role for aminopeptidases from *Lactococcus lactis, Escherichia coli and Salmonella* in protein turnover has been established. *L. lactis* grows in milk, which has limiting amounts of peptides and amino acids. Proteases digest casein from milk to provide required amino acids and nitrogen for metabolism (Gonzales and Robert-Baudouy, 1996). In *E. coli* and *S. typhimurium*, during log phase growth, 1 – 2 % of cellular protein is recycled, and that amount increases to 5 - 12 % during the nutrient limiting environment of stationary phase growth (Lazdunski, 1989). *E. coli* and *S. typhimurium* aminopeptidase mutants showed decreased protein degradation, decreased protein synthesis and decreased viability during growth in nutrient limiting environments (Reeve *et al.*, 1984; Yen *et al.*, 1980) suggesting a role for these proteases in nutrient acquisition.

Recycling of amino acids from culture filtrate proteins has been reported by Reich *et al.* (1981) suggesting a similar role for mycobacterial proteases in growth. Interestingly, one of the effects of growing *M. tuberculosis* in zinc deficient media, an environment that may lead to reduced or inactivity of our aminopeptidase, is an accumulation of protein in the culture supernatant (DeBruyn *et al.*, 1981). The aminopeptidase reported here may play a role in that recycling process providing a source of amino acids for re-incorporation into tuberculoproteins.

Finally, aminopeptidases may play a role in determining the lifetime of proteins. The relationship between the N-terminal amino acid of a protein and its half-life in *E. coli* was described (Bachmair *et al.*, 1986). The N-terminal amino acid determines how long a protein will remain undegraded and ranges from minutes to hours. Although a role for amino acid transferases in this process has been proven in mutant

studies, the potential role for aminopeptidases in this process should be examined (Bachmair *et al.*, 1986). Bacterial aminopeptidases could contribute to the regulation of protein turnover.

IV.8.b. Role in pathogenesis

A mycobacterial aminopeptidase may play a role in preventing acidification of the phagosome after internalization of the bacteria into the macrophage. *M. tuberculosis* growth rate is extremely slow with a doubling time of 18 hours under optimal conditions (Wayne, 1994). The rate-limiting step in growth is nucleic acid synthesis (Wheeler and Ratledge, 1994). The aminopeptidase may provide surplus amino acids, that is, amino acids which are not required for re-incorporation into tuberculoproteins. The fate of surplus amino acids includes the removal of the amino group for production of ammonia (Stryer, 1995b). Additional enzymes found in *M. tuberculosis* which may contribute to ammonia production include L-arginase, L-asparaginase, L-glutaminase and urease (Gordon *et al.*, 1980; Jayaram *et al.*, 1968; Clemens *et al.*, 1995) all of which can use amino acids or their catabolites as substrates. The mycobacterial aminopeptidase may provide the first step in protein or peptide catabolism for the production of ammonia by the bacterium.

Although the aminopeptidase was isolated from an avirulent strain of the organism (H37Ra), the *pepD* gene has been identified in the genome sequence of the virulent *M. tuberculosis* strain H37Rv (Cole *et al.*, 1998). The predicted *pepD* gene product shares N-terminal amino acid homology with our protein and is predicted from homology to other aminopeptidases to code for a neutral aminopeptidase activity similar to that which we have found. The differences between strains H37Rv and H37Ra have not been completely defined at a molecular level. There are additional cellular requirements for the production of ammonia by the organism. These include transport of proteins or peptides into the cell, if the degradative proteases are

intracellular, and enzymes for further catabolism of the amino acids produced by the aminopeptidase. If any of the proteins involved in these processes are absent or dysfunctional in *M. tuberculosis* strain H37Ra, that could account for, or contribute to, its avirulence.

IV.9. Summary

A mycobacterial aminopeptidase may play a role in bacterial growth by providing a source of amino acids for re-incorporation into proteins or for further breakdown to provide N, C and energy sources for metabolism. It may also play a role in facilitating survival of the *M. tuberculosis* inside a macrophage by providing amino acids for further catabolism to ammonia, thereby preventing acidification of the phagosome and phagosome-lysosome fusion. Considering its potential roles in both survival and pathogenesis, it represents a target for chemotherapeutic agents.

Chapter V. Future Studies

An extraordinary amount of information about *Mycobacterium tuberculosis* has been provided recently by sequencing the H37Rv genome (Cole *et al.*, 1998). Along with the advent of molecular biology tools that can be used for the organism, we believe there will be a dramatic change in the way we approach the protein science of the organism. The presence of a predicted open reading frame does not prove that a gene or gene product exists. Further, the expression of a gene product *in vitro* does not address its relevance *in vivo*. It may be neither produced nor functional as predicted. To address these questions, we must study the proteins expressed by the organism in relevant environments. This approach could be used in future studies of the *M. tuberculosis* proteases.

There are 29 gene products presently predicted to produce proteases or protease subunits from the *M. tuberculosis* H37Rv genome sequence (Cole *et al.*, 1998). The information provided by the genome sequence could be used in two ways. First, peptides mimicking predicted surface exposed protease epitopes could be synthesized. These could be used to prepare antibodies specific for each protease providing powerful tools for the purification of the enzymes from *M. tuberculosis* (Bulinski, 1986). Enzymes could then be purified from *M. tuberculosis* and characterized. Further, antibodies to synthetic peptides have been used to identify regions essential for activity in the *Pseudomonas aeruginosa* elastase (Kooi *et al.*, 1997). Advantages of this approach are the ability to characterize the active site using anti-peptide antibodies to the predicted active site region and obtaining the active protease. Disadvantages are the potential cross-reactivity between anti-protease antibodies raised against active sites and other *M. tuberculosis* protease active sites, the slow growth rate of *M. tuberculosis* for preparation of starting material, and the potentially low amount of the protease produced.

Secondly, predicted proteases could be cloned by PCR and expressed in *E. coli*. Successful expression of *M. tuberculosis* gene products has been achieved in *E. coli* using both λgt11 and plasmid vectors (Shinnick *et al.*, 1995). The advantage of working in *E. coli* is that growth and production of starting material for purification will be faster than in *M. tuberculosis* and expressed proteins may constitute up to 30 % of cellular protein as was shown for the catalase-peroxidase protein (Nagy *et al.*, 1997). The disadvantages of this technique are that large amounts of expressed protease may be toxic to *E. coli* and may not be expressed in an active form. The expression of a single subunit of a multi-subunit protease would not result in expression of activity. Purified proteases could be used to determine their crystallographic structure or to screen drugs, which may be active against them, and therefore, may be useful as antimycobacterial chemotherapeutics.

Synthetic protease substrates, which mimic different substrates, could be used to more precisely define the substrate specificity of purified proteases. The elucidation of substrates for each of the cloned proteases may lead to additional hypotheses regarding their potential role in the human host.

Researchers have successfully replaced *M. bovis* and *M. tuberculosis* genes with defective genes by allelic exchange (Pelicic *et al.*, 1996; Balasubramanian *et al.*, 1996; Pelicic *et al.*, 1997). The gene of interest is mutated by the addition of a kanamycin resistance cassette and then DNA containing the mutant gene is electroporated into *M. tuberculosis*. Counterselection for the electroporated DNA is performed either by a counterselectable marker on a plasmid (Pelicic *et al.*, 1996; Pelicic *et al.*, 1997) or by the inability of the electroporated DNA to sustain itself (Balasubramanian *et al.*, 1996). Pelicic *et al.* (1997) used the *B. subtilis sacB* gene under the induction of a temperature sensitive promoter in *M. tuberculosis* for counterselection. Counterselection of the plasmid DNA was performed by growing

the organisms on 2 % sucrose at non-permissive temperatures forcing recombination with the defective gene. They achieved 100 % allelic exchange mutants during their replacement of the *purC* gene in *M. tuberculosis*. Illegitimate recombination can occur between the kanamycin cassette and the bacterial chromosome. Balasubramanian *et al.* (1996) found that only 6 % of their transformants were the result of the desired recombination event. It is therefore necessary to screen for legitimate recombination events genetically. I propose that this method could be used to create defective allelic exchange mutants for the predicted protease gene products to examine their contribution to *M. tuberculosis* survival and pathogenesis.

The two hypotheses that we have proposed for the role of proteases in *M. tuberculosis* could be tested in *in vitro* assays with defective protease mutants created by allelic exchange. To examine the role of proteases in nutrient acquisition, growth would be evaluated for wild type and mutant strains providing protein as the sole source of amino acids for growth. Sauton's medium contains L-asp (Atlas, 1993) and this would be replaced with bovine serum albumin for the experiment. Decrease in growth rate of a mutant in the modified medium versus the L-asp-containing medium compared to wild type *M. tuberculosis*, or its inability to grow, would indicate a role for the wild type protein in growth.

Yen *et al.* (1980) demonstrated that aminopeptidases were required for recycling of protein by examining the location of radiolabeled leucine provided during microbial growth. All of the radiolabel appeared in proteins which were large enough to be excluded by Sephadex G25 in wild type *S. typhimurium* while peptidase mutants contained a large pool of material which could enter the column. This demonstrated that peptidase mutants accumulate small oligopeptides rather than recycling amino acids for protein synthesis (Yen *et al.*, 1980). *M. tuberculosis* wild type and allelic exchange protease mutants could be grown in the presence of radiolabeled leucine and

the location of the radiolabeled leucine could be determined. Similar results would show a role for a protease in the recycling of culture filtrate proteins demonstrated by Reich *et al.* (1981).

To examine the role of proteases in prevention of acidification of the phagosome, the pH of phagosomes infected with wild type or mutant strains would be assessed as described by Crowle *et al.* (1991). Localization of a weak base, DAMP, which accumulates in acidic compartments of the macrophage, can be detected by colloidal gold labeled α-dinitrophenol antibodies, which bind to it. Alternatively, Sturgill-Koszycki *et al.* (1994) demonstrated the prevention of acidification of macrophages after infection with *M. avium* over a 90 min time course by spectrofluorometry of NHS-carboxyfluorescein-labeled particles, a pH sensitive fluorescent agent. Acidification of the phagosome in a mutant strain would indicate a role for the specific mutant protease in prevention of acidification of the phagosome.

The genetic information available for *M. tuberculosis* and the recent advances in the utilization of genetic tools in this organism, provide us with a means to screen specific protease genes elucidating their role in survival and pathogenesis of the organism.

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Collagenase Appendix. Partial Purification And Characterization Of The *M. tuberculosis* Collagenase

Collagenase Appendix. Section I. Materials and Methods

Materials and methods, which differ from those used during the purification and characterization of the aminopeptidase, are included in this section. The bacterial strain used, media and culture conditions and production of ECF and sonicate are identical to those in Chapter II.

A.I.1. Assay for collagenase

Collagenase was assayed using N-succinyl-gly-pro-leu-gly-pro-alpha-methylcoumarin (N-succinyl-GPLGP-AMC; Kojima *et al.*, 1979; Sigma, St. Louis, MO, USA) as a substrate. The substrate was dissolved in DMSO to a concentration of 50 mg/mL and then diluted 1/ 1000 in phosphate buffered saline (PBS) pH 7.4 for use in the assay. Fifty µl of enzyme solution to be assayed was pipetted into a 96-well polysorp nunc-immuno plate (Nalge Nunc International, Rochester, NY, USA) and 150 µl of assay solution was added.

A.I.2. Purification of collagenase

Column purifications were performed using the FPLC Basic System (Pharmacia Biotech, Uppsala, Sweden). Columns used were the HILoad Q Sepharose Fast Flow column (26 mm x 10 cm) for anion exchange chromatography, Phenyl Sepharose 6FF (low sub) column (0.7 mm x 2.4 cm) for hydrophobic interaction chromatography and the Superdex 200 HR 10/30 gel filtration column (10 mm x 30 cm) for size exclusion chromatography (Pharmacia Biotech, Uppsala, Sweden).

A.I.2.a. Q Sepharose Fast Flow anion exchange chromatography

The Q Sepharose Fast Flow column was run identically to that described in section II.6.a. It was used to separate the aminopeptidase and collagenase activities.

An alternative gradient was also run by loading 40 mL of sonicate onto the Q Sepharose Fast Flow column and eluting with a single step followed by a linear gradient. The column was washed with 100 mL of 0.3 M NaCl in 20 mM Bis-tris pH 6.5 and then a gradient from 0.30 to 0.55 M NaCl was run. Finally, the column was washed with 1.0 M NaCl in the same buffer.

A.I.2.b. Phenyl LO SUB hydrophobic interaction chromatography

A 2 M ammonium sulfate solution (58 mL) was added to 58 mL of the peak pool drop wise while stirring to bring the ammonium sulfate concentration to 1.0 M. The protein suspension was centrifuged at 20,000 x g for 30 min at 4°C to remove any precipitate. The resulting protein suspension was loaded on the 1 mL Phenyl LO SUB column which had been pre-equilibrated with 50 mM sodium phosphate pH 7.0, 1.0 M ammonium sulfate. The column was run at 1 mL min^{-1} collecting 2 mL fractions. The column was washed with 10 column volumes of start buffer and then a 50 mL gradient was run from 1.0 M to 0.0 M ammonium sulfate in 50 mM sodium phosphate pH 7.0. The column was washed with 10 column volumes of 50 mM sodium phosphate pH 7.0. The pooled fractions with highest activity (54 mL) were concentrated to 0.5 mL in an amicon ultrafiltration cell (Amicon Inc., Beverly, MA, USA) using a membrane with a molecular weight cut off of 10,000 daltons and then by microconcentration in a centricon 10 (Amicon Inc., Beverly, MA, USA) at a speed of 5,000 x g at 4°C.

A.I.2.c. Superdex 200 size exclusion chromatography

The Superdex 200 size exclusion chromatography column was run identically to that described in section II.6.c.

A.I.3. Characterization of the crude and partially purified collagenase activity

A.II.3.a. Inhibition of the collagenase activity

Additional inhibitors tested for the collagenase activity were TPCK and TLCK. They were dissolved in water and used at a final concentration of 0.1 mM.

Collagenase Appendix. Section II. Results

A.II.1. *Mycobacterium tuberculosis* growth and collagenase production

The growth of *Mycobacterium tuberculosis* was measured weekly for 6 weeks by reading the A600 of the culture. Extracellular filtrate (ECF) and sonicate were prepared from an aliquot of the culture at each time point and assayed for the amount of collagenase activity present (FIG. A-1a). Activity was detected in both ECF and sonicate at 7 days post inoculation. The amount of activity in sonicate increased for 6 weeks but the amount produced increased more slowly after 4 weeks. The amount of activity present in ECF increased for 6 weeks with a larger increase in the amount detected during weeks 1 - 4. The amount of activity per culture growth, estimated from the A600 of the culture at each time point, is shown in FIG. A-1b. The ratio of enzyme activity in sonicate to culture growth increased slowly for the first 3 weeks and then leveled off for weeks 4, 5 and 6. The ratio of collagenase activity in ECF to culture growth was constant throughout the 6 weeks.

A.II.2. Partial purification of the *M. tuberculosis* collagenase

Collagenase activity was purified more than 1,000 fold from 4 week ECF with a net yield of 40 % of enzyme activity (Table A-1).

A.II.2.a. Q Sepharose Fast Flow anion exchange chromatography

The purification achieved by the Q Sepharose Fast Flow column was approximately 30 fold with a yield of 100 % of activity. The peak of activity eluted at a salt concentration of 0.37 M NaCl in 20 mM Bis-tris pH 6.5. The salt concentration of the fractions with maximum activity ranged from 0.33 - 0.47 M NaCl (FIG. A-2a).

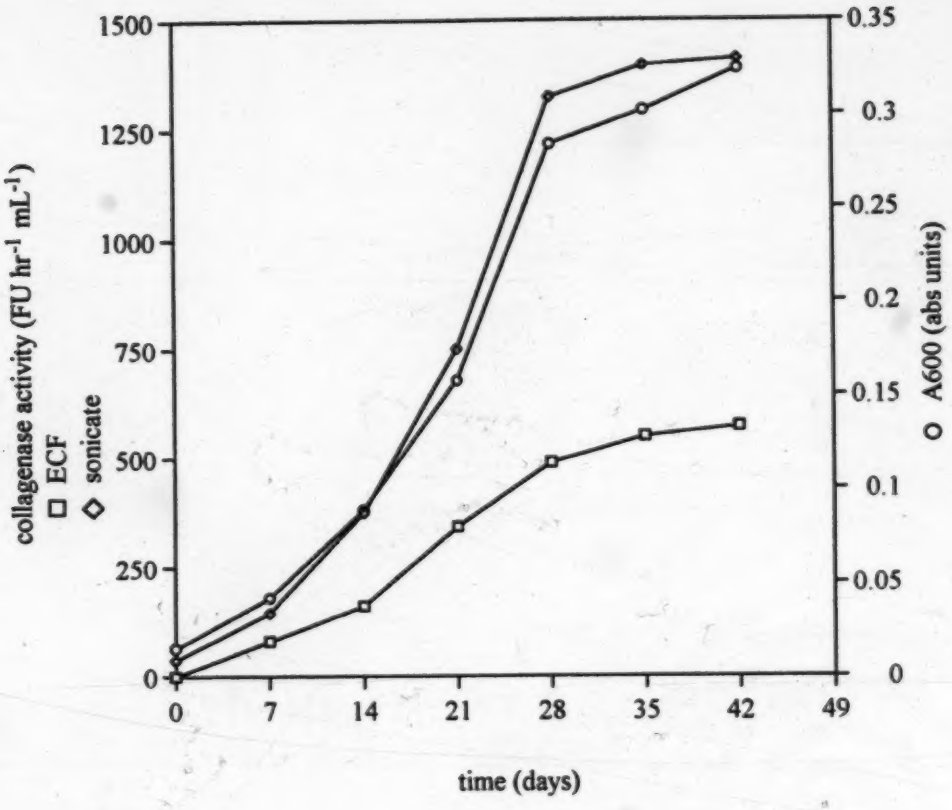

FIG. A-1a. Amount of collagenase activity in extracellular filtrate (ECF) and sonicate measured at weekly intervals for 6 weeks. Activity was measured by reading the fluorescence produced upon digestion of the synthetic substrate N-succinyl-GPLGP- α-methylcoumarin. The number of cells present was estimated by reading the A600 (absorbance at 600 nm) of the culture at each time point. 1FU = 0.34 pmoles AMC.

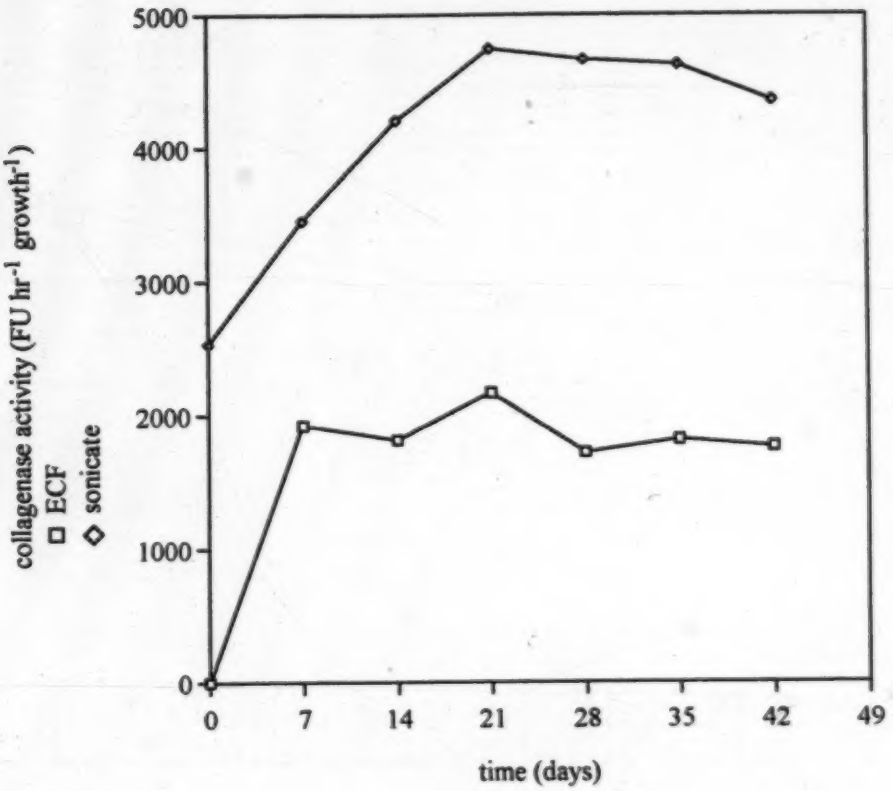

FIG. A-1b. Amount of collagenase activity in extracellular filtrate (ECF) and sonicate per culture growth measured at weekly interval for 6 weeks. Activity was measured by reading the fluorescence produced upon digestion of the synthetic substrate N-succinyl-GPLGP- α-methylcoumarin. The culture growth was estimated by reading the A600 (absorbance at 600 nm) of the culture at each time point. 1 FU = 0.34 pmoles AMC.

TABLE A-1. Summary of collagenase partial purification

sample[a]	protein (mg mL^{-1})[b]	specific activity (FU hr^{-1} mg^{-1})[c]	net purification (fold)	net yield (%)
conc ECF	2.06	11,800	-	100.0
Q Seph FF	0.35	344,000	29.0	100.0
Phe LO SUB	2.25	1,230,000	104	42.3
Superdex 200	0.068	12,400,000	1,050	38.6

[a] Abbreviations: conc ECF, concentrated extracellular filtrate; Q Seph FF, anion exchange chromatography on a Q Sepharose Fast Flow column; Phe LO SUB, hydrophobic interaction chromatography on a low substituted phenyl sepharose column; Superdex 200, size exclusion chromatography on a Superdex 200 column. Samples are the product of the purification procedure listed.

[b] Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA, USA).

[c] Collagenase activity was assayed by measuring the increase in fluorescence in one hour due to the release of α-methylcoumarin (AMC) from the synthetic substrate, N-succinyl-GPLGP-AMC as described in section A. I.1. 1 FU = 0.34 pmoles AMC.

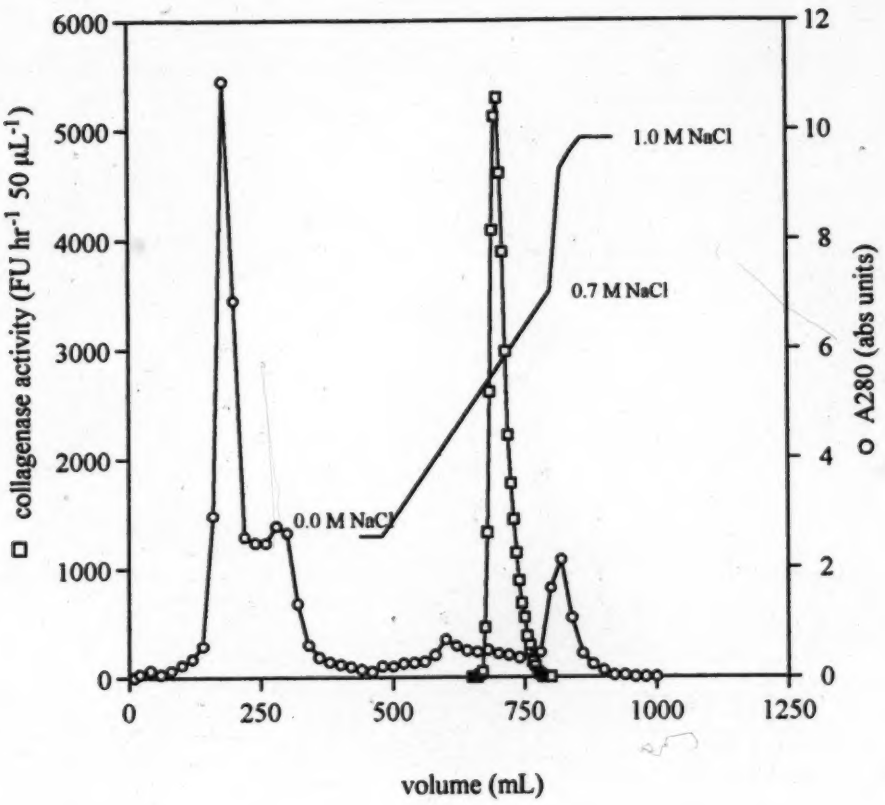

FIG. A-2a. Profile of collagenase elution from the Q Sepharose FF anion exchange chromatography column. The peak of protease activity eluted at a salt concentration of 0.37 M NaCl in 20 mM Bis-tris buffer pH 6.5. Salt concentration present in fractions was measured by reading the conductivity of fractions and is shown by a solid line on the graph. 1 FU = 0.34 pmoles AMC released from the synthetic substrate, N-succinyl-GPLGP-AMC; A280 = absorbance at 280 nm.

The volume of the peak of activity was 75 mL. The collagenase activity was concentrated 4.9 fold over the initial material loaded (Table A-1).

FIG. A-2b shows elution of the aminopeptidase and collagenase from the same column using a different salt gradient. By washing the column with 2 column volumes of 0.3 M NaCl in 20 mM Bis-tris pH 6.5 prior to running a gradient from 0.3 - 0.6 M NaCl, it is possible to elute collagenase activity, which is free of aminopeptidase activity. Aminopeptidase activity washes through the column in the 0.3 M NaCl wash while collagenase activity elutes in the early portion of the gradient.

A.II.2.b. Phenyl LO SUB hydrophobic interaction chromatography

The purification achieved by the Phenyl LO SUB column was approximately 3.6 fold with a yield of 42 % of activity. The collagenase peak eluted at a salt concentration of 0.56 M ammonium sulfate in 50 mM sodium phosphate pH 7.0. The salt concentration of the fractions pooled ranged from 0.80 – 0.14 M ammonium sulfate and was in a total volume of 69 mL (FIG. A-3). This volume was concentrated by amicon filtration to 0.5 mL resulting in a concentration of 23 fold over the initial material loaded (Table A-1).

A.II.2.c. Non-denaturing Superdex 200 size exclusion chromatography column

The purification achieved by non-denaturing size exclusion chromatography was approximately 10 fold with a yield of 91 % (Table A-1). The peak of collagenase activity eluted from the Superdex 200 column at a volume of 14.4 $\pm$ .1 mL after loaded material entered the column. There were four fractions (0.5 mL each) which contained more than 10 % of the activity of the peak (FIG. A-4).

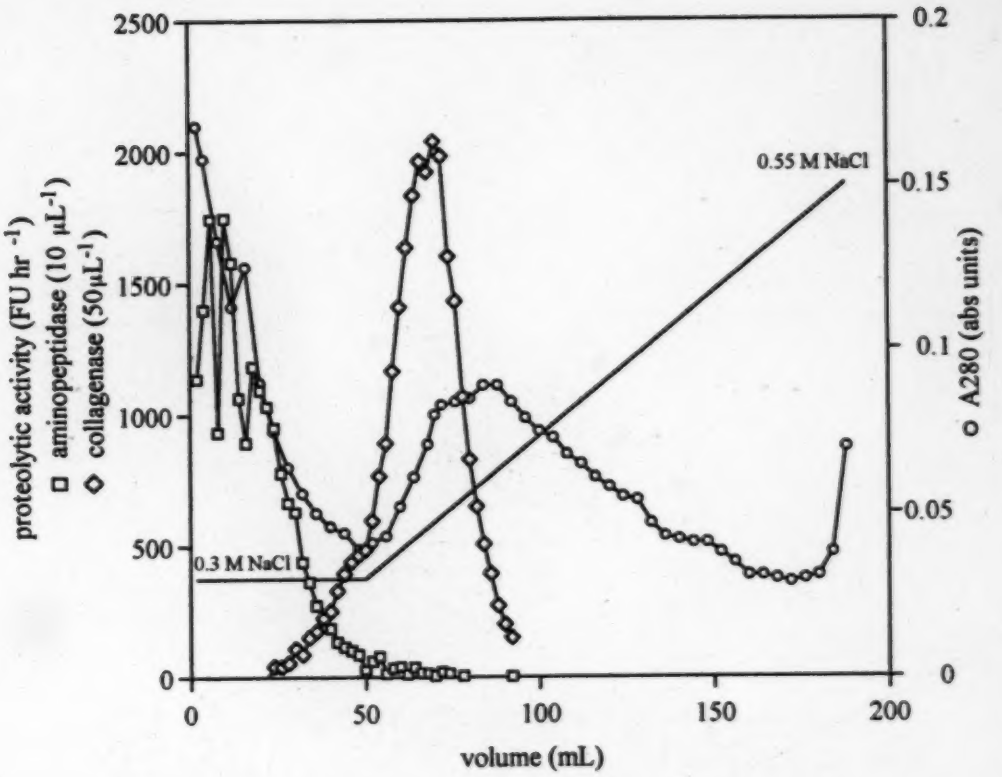

FIG. A-2b. Profile of aminopeptidase and collagenase activity elution from Q Sepharose Fast Flow anion exchange column under different elution conditions. The portion of the salt gradient shown is represented by a solid black line. 1 FU = 0.34 pmoles AMC released from the synthetic substrate, AAF-AMC, for aminopeptidase detection or N-succinyl-GPLGP-AMC, for collagenase detection; A280 = absorbance at 280 nm. Washing the column with 0.3 M NaCl in 20 mM Bis-tris pH 6.5 caused aminopeptidase activity to flow through the column in the initial wash fractions and allowed the collagenase activity to be recovered almost free of aminopeptidase contamination.

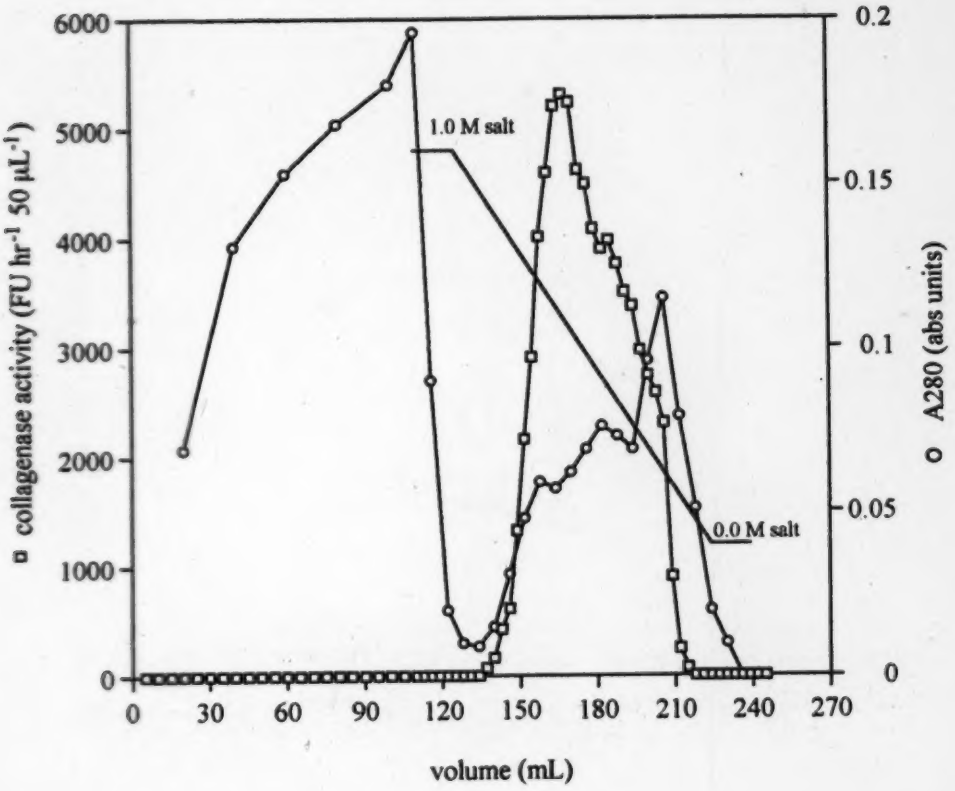

FIG. A-3. Profile of collagenase elution from the Phenyl LO SUB hydrophobic interaction chromatography column. The peak of protease activity eluted at a concentration of 0.56 M ammonium sulfate in 50 mM sodium phosphate buffer pH 7.0. Salt concentration present in fractions was measured by reading the conductivity and is shown by a solid line on the graph. 1 FU = 0.34 pmoles AMC released from the sythetic substrate, N-succinyl-GPLGP-AMC; A280 = absorbance at 280 nm.

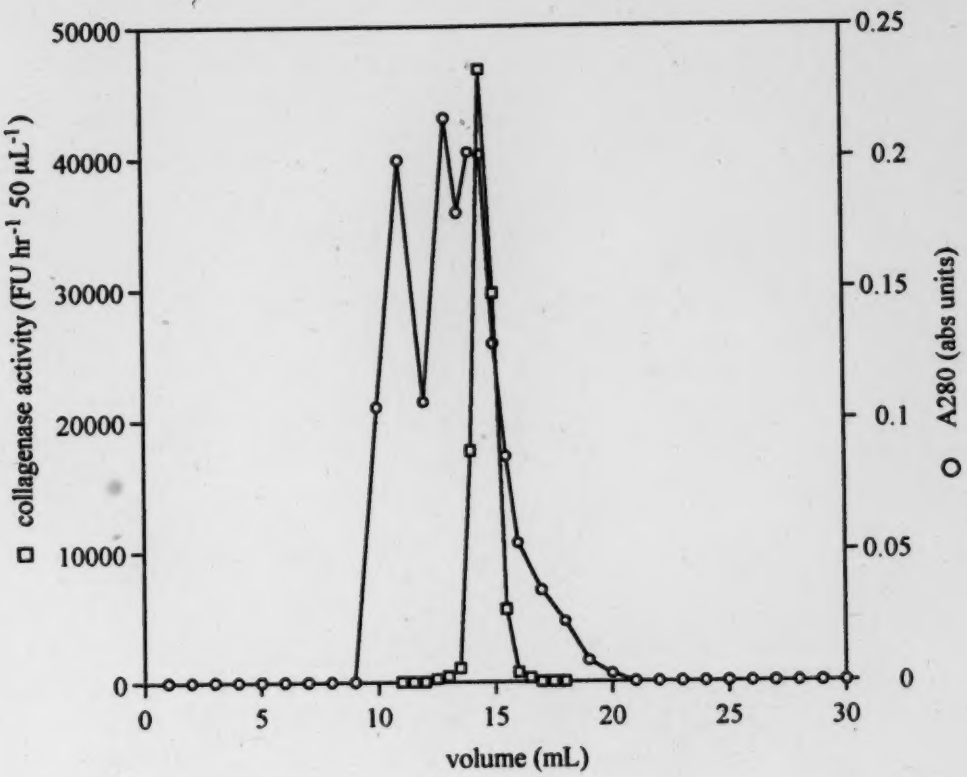

FIG. A-4. Profile of collagenase elution from non-denaturing Superdex 200 size exclusion chromatography column. 1 FU = .34 pmoles AMC released from the synthetic substrate, N-succinyl-GPLGP-AMC; A280 = absorbance units. The peak of protease activity eluted at a volume of 14.4 $\pm$ .1 mL corresponding to a molecular weight of 53 $\pm$ 6 kilodaltons for the native protein.

FIG. A-5 shows an SDS-PAGE analysis of the purification achieved by Superdex 200 column chromatography. The peak of activity occurred in the fraction represented in lane 7 and activity was present in each of the fractions run in lanes 6 – 8.

A.II.3. Characterization of the purified protein

A.II.3.a. Determination of the molecular weight of non-denatured collagenase by Superdex 200 size exclusion chromatography

FIG. III-10 shows the linear relationship of the log of the molecular weight of known molecular weight standards versus their elution volume from the Superdex column run under the same, non-denaturing conditions as the partially purified collagenase. The peak of protease activity eluted at a volume of 14.4 $\pm$ .1 mL from the Superdex 200 column corresponding to a molecular weight of approximately 53 $\pm$ 6 kDa from the standard curve.

A.II.3.b. Determination of the isoelectric point of the partially purified collagenase

The isoelectric point of the collagenase was determined using a chromatofocusing column and eluting with polybuffers diluted 1 in 10 pH 3.0. (FIG. A-6). The pI of this protein by chromatofocusing was 3.4 $\pm$ 0.2.

A.II.4. Characterization of the collagenase activity

A.II.4.a. pH optimum of collagenase activity

Collagenase activity was optimal at a pH of 8.5 (FIG. A-7). It maintained more than 50 % optimal activity from pH 6.5 to 9.5 and still displayed detectable

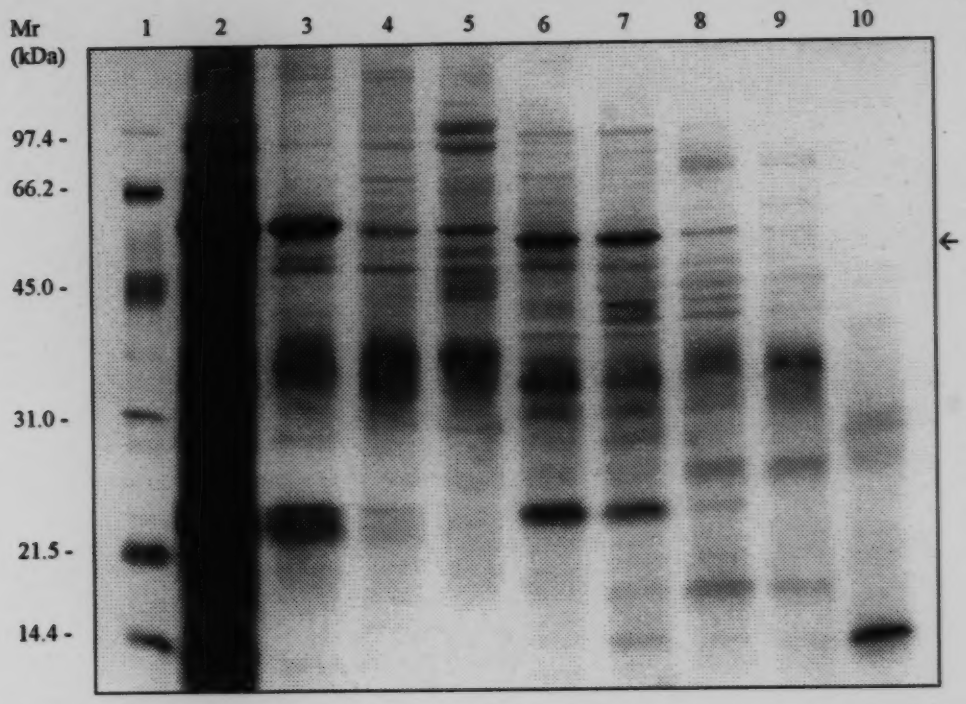

FIG. A-5. Silver stained SDS-PAGE analysis of collagenase elution from non-denaturing Superdex 200 chromatography column. Low molecular weight markers: phosphorylase b (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (Mr 14,400; lane 1). Phe LO SUB column pool (150 μL, lane 2). Fractions are named as the volume at which they eluted after the Phe LO SUB column pool was loaded. 10.5 mL (150 μL, lane 3). 12.0 mL (150 μL, lane 4). 13.0 mL (150 μL, lane 5). 14.0 mL (150 μL, lane 6). 14.5 mL (150 μL, lane 7). 15.0 mL (150 μL, lane 8). 16.0 mL (150 μL, lane 9). 17.0 mL (150 μL, lane 10). Mr = molecular weight; kDa = kilodaltons. The peak of collagenase activity eluted at a volume of 14.4 ± .1 mL (lane 7) corresponding to a molecular weight of 53 ± 6 kDa. The arrow shows the position corresponding to a molecular weight of 53 kDa.

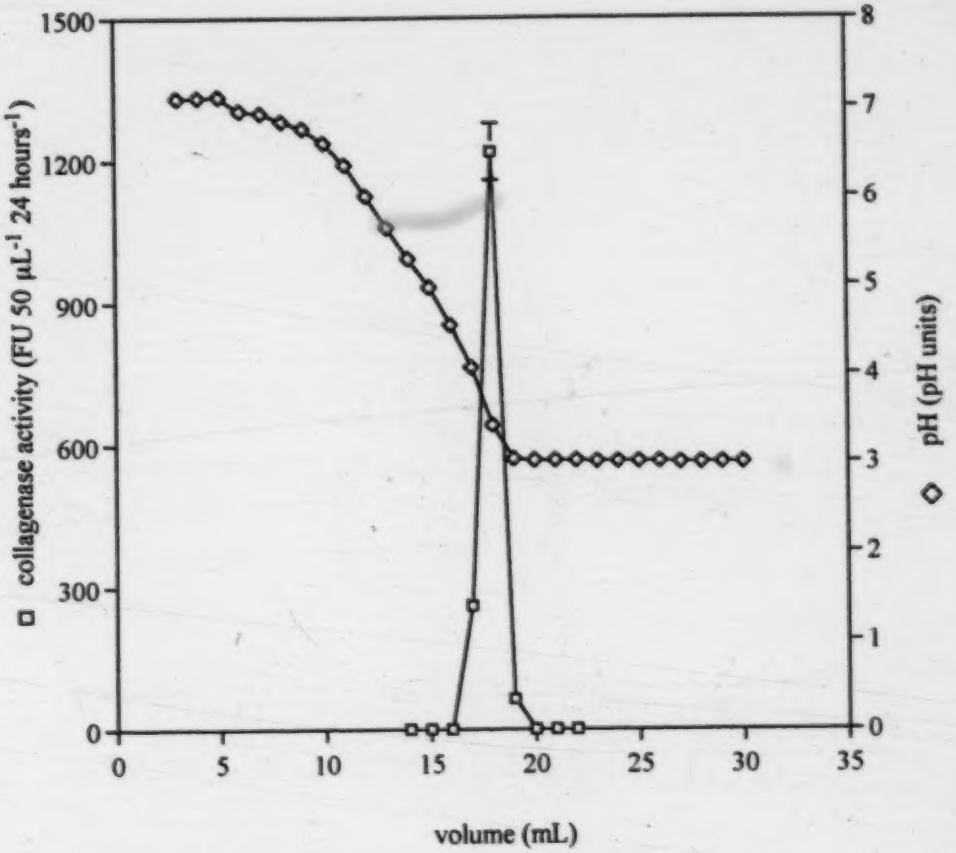

FIG. A-6. Determination of the isoelectric point of the partially purified collagenase. Partially purified protease (.01 mg) was loaded on a chromatofocusing column and eluted with a 1/10 dilution of polybuffer 74 pH 3.0 with iminodiacetic acid. 1 FU = 0.34 pmoles AMC released from the synthetic substrate, N-succinyl-GPLGP-AMC. The pI of the partially purified collagenase was 3.4. Standard deviations are calculated for n=3.

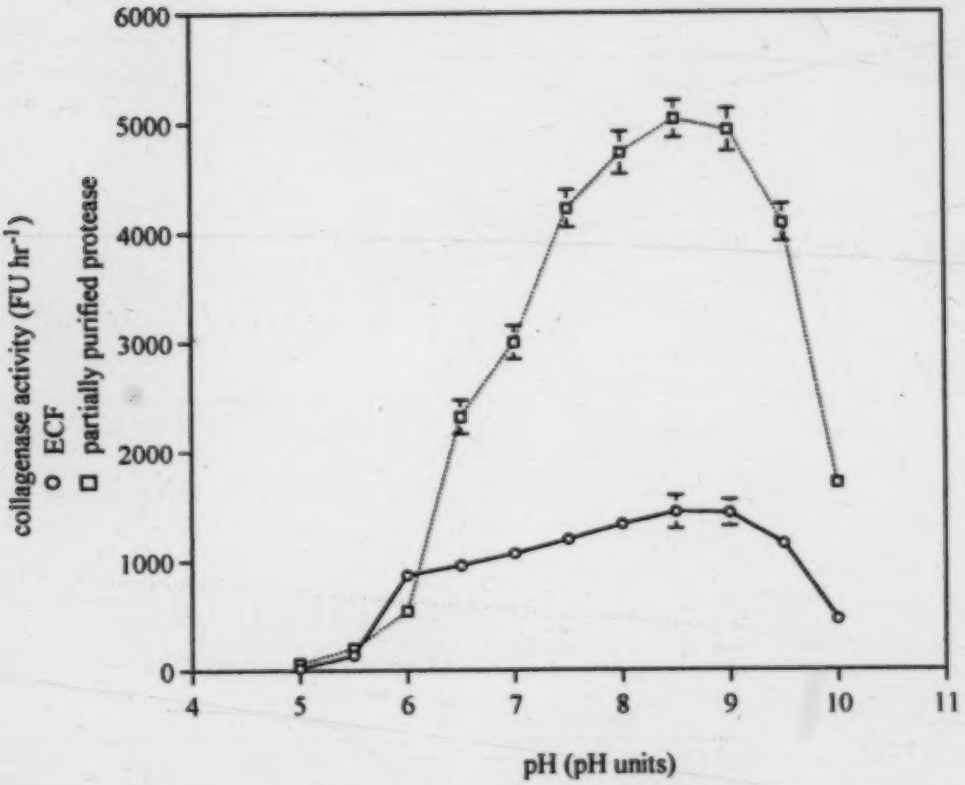

FIG. A-7. Effect of pH on collagenase activity. Activity of extracellular filtrate (ECF; 0.1 mg) and partially purified protease (0.34 mg) were assayed at different pH values. 1 FU = 0.34 pmoles AMC released from the synthetic substrate, N-succinyl-GPLGP-AMC. Standard deviations are calculated for n=3.

activity at pH 5.5. ECF and partially purified protease showed similar pH optima for collagenase activity.

A.II.4.b. Temperature optimum of collagenase activity

The collagenase temperature optimum was 34°C and 37°C for crude ECF and partially purified collagenase, respectively (FIG. A-8). More than 25 % of activity was retained at 20°C and more than 50 % of activity was retained at 48°C. Activity was reduced at temperatures ≥ 56°C.

A.II.4.c. Inhibition of collagenase activity

Collagenase activity in ECF and partially purified protease was determined in the presence of protease inhibitors to determine the class of protease. 3,4-DCI and PMSF fully inhibited both ECF and partially purified collagenase (Table A-2). Enzyme activity was not inhibited by TPCK, TLCK, leupeptin, EDTA, E-64 or pepstatin (Table A-2).

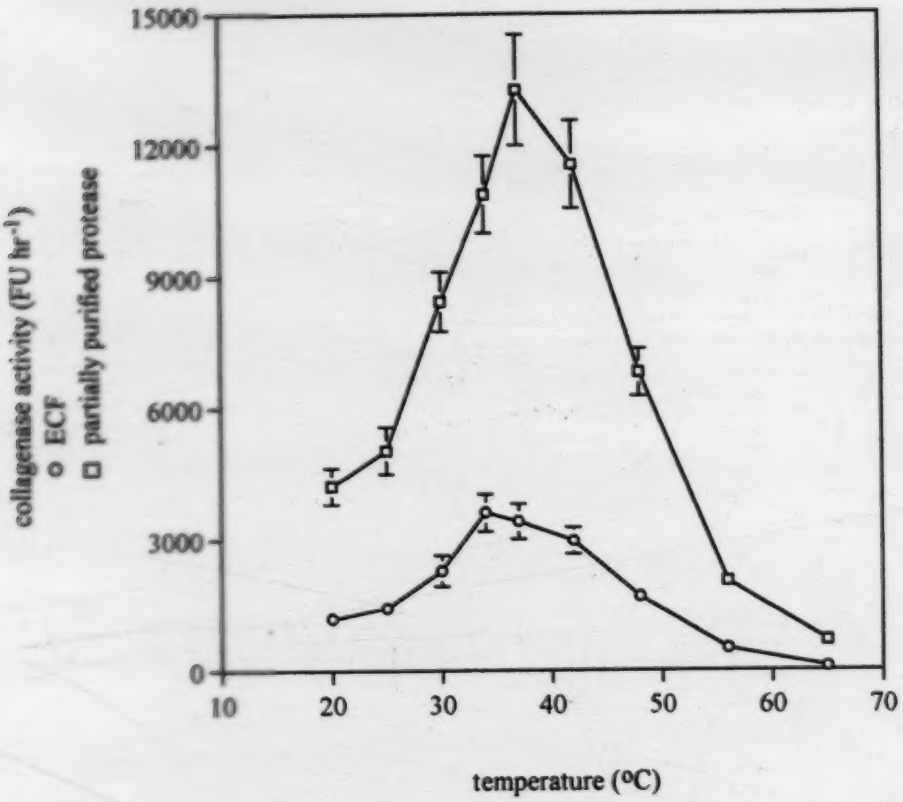

FIG. A-8. Effect of temperature on collagenase activity in extracellular filtrate (ECF; 0.1 mg) and partially purified protease (0.34 μg). 1 FU = 0.34 pmoles AMC released from the synthetic substrate, N-succinyl-GPLGP-AMC. Standard deviations are calculated for n=3.

TABLE A-2. Effects of protease inhibitors on collagenase activity

inhibitor[b]	concentration (mM)	inhibition of collagenase activity (%)[a]	
		ECF[c]	partially purified protease
3,4-DCI	0.10	100.0 ± 0.0	99.6 ± 0.4
E-64	0.01	0.3 ± 6.9	0.3 ± 0.6
EDTA	10.0	7.7 ± 2.3	0.2 ± 0.3
leupeptin	0.10	11.5 ± 6.2	4.7 ± 4.2
pepstatin	0.001	6.0 ± 8.5	0.5 ± 0.8
PMSF	1.0	99.8 ± 0.3	100.0 ± 0.0
TPCK	0.1	2.5 ± 2.2	2.8 ± 2.0
TLCK	0.1	0.4 ± 0.2	0.2 ± 0.1

[a] The effects of protease inhibitors on enzyme activity in concentrated ECF and purified enzyme were measured. Results are presented as the % inhibition of collagenase activity and standard deviations are calculated for n=3.

[b] Descriptions of the effective inhibitors are provided in Table IV-1.

[c] ECF = extracellular filtrate concentrate.

Collagenase Appendix. Section III. Discussion

A.III.1. Partial purification of the collagenase

The *M. tuberculosis* H37Ra collagenase has been purified more than 1,000 fold with a yield of approximately 40 %. However, there are still a large number of protein bands present in the partially purified protein mixture.

Electroelution of individual bands from the Superdex 200 column pool results in a loss of collagenase activity. It is possible that the effect of SDS on the collagenase is irreversible (Dong *et al.*, 1997) or that the collagenase is a multi-subunit protease in which subunits are being separated by this procedure. Complete removal of SDS and reconstitution of collagenase activity could be attempted by other procedures (Dong *et al.*, 1997).

It is also possible that the substrate used to follow the isolation of the collagenolytic activity, N-succ-GPLGP-AMC, is not ideal for the purified enzyme(s) involved. If more than one protease were required for the activity observed, separation of those proteases would result in a loss of activity. That is, the substrate may be cleaved internally, and then the AMC group released by a pro dipeptidyl aminopeptidase or by an iminopeptidase (Cunningham and Connor, 1997). A more appropriate substrate for these activities would be an unblocked G-P-AMC or P-AMC, respectively. The aminopeptidase activity that we describe in *M. tuberculosis* has low activity against each of these substrates, but can be separated from the collagenase activity by Q Sepharose Fast Flow chromatography (FIG. A-2b).

A.III.2. Characterization of the collagenase protein and its activity

The non-denatured collagenase has a molecular weight of 53 ± 6 kDa by size exclusion chromatography and a pI of 3.4 by chromatofocusing.

The pH optimum of activity is alkaline at pH 8.5 for both ECF and partially purified collagenase. Activity is present over a wide pH range, from 6 – 10. The temperature optimum is 34°C and 37°C for ECF and partially purified collagenase, respectively.

Serine protease inhibitors, 3,4-DCI and PMSF, effectively inhibit collagenase activity in both ECF and partially purified protease. Cysteine and aspartic protease inhibitors did not inhibit activity. The divalent metal chelator, EDTA, also did not inhibit activity. This indicates that the collagenase has a serine active site and does not require metal cations for activity.

The class of protease provides us with additional information that can be applied to the purification procedure. Serine protease-specific affinity column matrices, arginine-sepharose and benzamidine-sepharose, are available commercially (Pharmacia Biotech, Uppsala, Sweden). Also, diisofluorophosphate (DFP), a serine protease inhibitor which can be radiolabeled, has been used to detect proteases with serine active sites separated by SDS-PAGE (Balow *et al.*, 1986). This procedure could be used to identify the protein of interest from our Superdex 200 column pool. The band of interest could be separated from contaminants by SDS-PAGE and electroeluted.

A.III.3. Comparison with the previously identified collagenolytic activity

The protease purified by Takahashi (1957) may be different from the one

described here. There is little characterization done on the protease. The molecular weight of the non-denatured protease was 77 kDa by sedimentation equilibrium. The molecular weight of our native protease was 53 $\pm$ 6 kDa by size exclusion chromatography. The activity described by Takahashi (1957) cleaved the synthetic substrate Z-GPLGP on the C-terminal side of L or alternatively on the N-terminal side of G releasing a G-P dipeptide. The activity we describe cleaves the synthetic substrate N-succ-GPLGP-AMC on the C-terminal side of P releasing the AMC group. The proteases have different cleavage specificities. In addition, the existence of an endopeptidase, which can release the G-P dipeptide from their substrate, provides a mechanism by which our protease substrate may be cleaved internally. An additional proline dipeptidase would then be required to release the AMC group from our substrate.

A.III.4. Proline-recognizing proteases

Because of the unique structure of proline and the steric restrictions it places on protein folding, bacteria have developed many protease activities specific for degradation of proteins around this amino acid (Gonzales and Robert-Baudouy, 1996). These include proline endopeptidase (X_n-P$\blacktriangledown$$Y_n$), proline dipeptidyl aminopeptidase (X-P$\blacktriangledown$$Y_n$), proline iminopeptidase (P$\blacktriangledown$$Y_n$), aminopeptidase P ($X\blacktriangledown$P-$Y_n$) as well as C-terminal exopeptidases specific for proline (Walter *et al.*, 1980; Cunningham and Connor, 1997). Interestingly, with the exception of one, the only exopeptidases identified to date which have serine active sites, cleave on the C-terminal side of proline, either proline dipeptidases or proline iminopeptidase (Gonzales and Robert-Baudouy, 1996). The exception is a D-amino acid

aminopeptidase (Gonzales and Robert-Baudouy, 1996).

The high specificity of prolyl-cleaving enzymes may give them a specific role in regulation of biologically active proteins or peptides (Cunningham and Connor, 1997). They may function in determining lifetime and degradation rate of biologically important proteins (Yaron and Nader, 1993). There is a proline-rich protein described in *M. bovis*, which is important in delayed-type hypersensitivity in guinea pigs (Romain *et al.*, 1993). Sequencing of the *M. tuberculosis* H37Rv genome shows a statistically significant preference for amino acids coded by G + C rich codons including proline (Cole *et al.*, 1998). The proline-rich protein described and others present in mycobacteria, may be specifically regulated by proline endo- and exoproteases.

A.III.5. *Mycobacterium tuberculosis* serine proteases

There are eleven predicted proteases, or catalytic subunits, which are predicted to or possibly have serine active sites from their DNA sequence (Table IV-2; Cole *et al.*, 1998; Altschul *et al.*, 1990). In the case of clpP, clpP', and clpX, these are the predicted catalytic subunits of a multimeric protease. The length of the polypeptides that would be produced from the open reading frames is from 200 to 673 amino acids, resulting in proteases or subunits of approximately 20 to 75 kDa. One of the predicted serine proteases, Genbank designation 0840c, is predicted to be a proline iminopeptidase, by its homology with other iminopeptidases. Its length is 286 amino acids and its predicted molecular weight is 31.5 kDa. It would cleave an N-terminal proline from a peptide or protein and could release AMC from P-AMC. If internal cleavage of our synthetic peptide by an endoprotease provided an accessible

N-terminal proline, the iminopeptidase could contribute to the collagenase activity we observe. Other than the predicted iminopeptidase, none of the other serine proteases are predicted to have cleavage specificity on the C-terminal side of proline.

However, mechanisms of protease specificity remain to be elucidated and the DNA sequence has no predictive value in determining protease substrate specificity. There are 5 predicted monomeric serine proteases with molecular weights between 40 and 65 kDa. This molecular weight range for the denatured protein could include a protein with a native molecular weight of 53 $\pm$ 6 kDa. These predicted or possible serine proteases include Rv1223 (Mr 56.5 kDa), Rv0983 (Mr 46.4 kDa), Rv3671c (Mr 40.7 kDa), Rv3883c (Mr 45.1 kDa) and Rv38863 (Mr 55.6 kDa). Any of these predicted gene products could also encode the observed collagenase activity.

A.III.6. Summary

The protease or proteases involved in the cleavage of our collagen-mimicking substrate remain to be determined. The same techniques described in Chapter V. Future Studies, for cloning and expressing the proteases in *E. coli* and creating protease-specific mutants, provide an alternative approach to learning about the collagenase; its substrate specificity and its role in growth and survival of *M. tuberculosis*. Similarly, preparation of antibodies specific to peptides designed from the predicted protein sequence for each of the serine proteases could provide a valuable tool for identifying the protein band of interest and purification of the protease from *M. tuberculosis* or *E. coli*.

Appendix 2. Screening of procedures for use during the purification of the aminopeptidase and collagenase enzymes from *M. tuberculosis*.

The following appendix includes a flow chart for techniques which can be screened for use in the isolation of proteins highlighting those used during the purification of the *M. tuberculosis* aminopeptidase. Also included are ten tables of results of procedures screened for use in the purification of the aminopeptidase and collagenase enzymes. For each procedure, the yield and purification of enzyme activities were used to determine whether they would be included in our isolation scheme. If the procedure resulted in a high yield of enzyme activity ($\geq$ 70 %), the procedure was further refined to optimize the purification achievable from extracellular filtrate concentrate and finally, the starting material that would be used for that purification procedure.

Figure A2-1. Diagram of possible isolation procedures

for use during protein purification

and the ones used for the purification of the *M. tuberculosis* aminopeptidase[a,b]

protein property

charge — **ion exchange chromatography**

- cation exchange chromatography
  - native conditions
  - denaturing conditions
- **anion exchange chromatography**
  - **native conditions**
  - denaturing conditions
- chromatofocusing

- experimentally determine:
column matrix (Q Seph FF)
elution conditions
(0-.7 M NaCl in 50 mM Bis-tris pH 6.5)

⇓

hydrophobicity — **hydrophobic interaction chromatography (native conditions)** / reverse-phase chromatography (denaturing conditions)

phenyl- **butyl-** **octyl-**

- experimentally determine:
binding conditions
(0.5 M AS in 50 mM sodium phosphate pH 7.0)
elution conditions
(0.5 – 0.0 M AS in 50 mM sodium phosphate pH 7.0)

- experimentally determine:
column matrix
elution conditions (buffer pH)

⇓

size — **size exclusion chromatography** — **SDS-PAGE (denaturing)**

- **native conditions**
- denaturing conditions

- experimentally determine:
matrix (Superdex 200 column)
buffer (50 mM sodium phosphate pH 7.0, .15 M NaCl)

- **elute (electroelution)**
- *in situ* (zymography)

- experimentally determine:
restoration of native activity (if required)
(dialysis in PBS pH 7.4, 1mM $ZnCl_2$)

⇓

specific properties of the protein:
- binds metal cations (IMAC)
- binds affinity matrices (eg. arginine-, PAB-, gelatin- and heparin-Sepaharose)
- binds known or described antibodies (antibody cross-linked to Sepharose)

[a] Isolation procedures used during the purification of the *M. tuberculosis* aminopeptidase are displayed in bold text.

[b] abbreviations: Q Seph FF, Q Sepharose Fast Flow anion exchange chromatography column; NaCl, sodium chloride; phenyl-, phenyl-sepharose hydrophobic interaction chromatography column; butyl-sepharose, butyl-sepharose hydrophobic interaction chromatography column; octyl-, octyl-sepharose hydrophobic interaction chromatography column; AS, ammonium sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; $ZnCl_2$, zinc chloride; IMAC, immobilized metal affinity chromatography; PAB, para-aminobenzamidine.

Table A2-1. Anion exchange chromatography column screening

column[a]	buffer	elution[b]	aminopeptidase yield (%)	aminopeptidase purification (fold)	collagenase yield (%)	collagenase purification (fold)
DEAE	50 mM Tris pH 8.0	batch - 1M NaCl	78	-	89	-
DEAE	50 mM Tris pH 8.0	0-1 M NaCl	72	6.0	84	19
DEAE	50 mM Tris pH 8.0	0-.6 M NaCl	74	7.7	80	24
DEAE	50 mM Tris pH 8.0	.1-.5 M NaCl	74	7.9	80	25
Q Seph	50 mM Tris pH 8.0	0-1 M NaCl	85.4	13.2	100	44.6
Q Seph	20 mM Bis-tris pH 6.5	0-1 M NaCl	90.5	32.9	100	41.2
Q Seph	20 mM piperazine pH 5.7	0-1 M NaCl	93.6	22.8	100	34.6
Q Seph	20 mM Bis-tris pH 6.5	.3-.7 M NaCl	55.5	11.9	100	35.0
Q Seph	20 mM Bis-tris pH 6.5	0-.7 M NaCl	87.1	63.6	100	29.0

[a] abbreviations: DEAE, the anion exchange medium DEAE Sephadex A-25 from Pharmacia Biotech (50 mL total volume); Q Seph, Q Sepharose Fast Flow anion exchange chromatography column (55 mL total volume); ECF, extracellular filtrate concentrate.

[b] Columns for screening were loaded with 20 mL of ECF, washed with 3 column volumes of starting buffer, the gradient was run at 2 mM/mL and the final wash volume was 3 column volumes.

[c] Pools contained fractions with greater than or equal to 10 % of the activity in the peak fraction.

Table A2-2. Separation based on isoelectric point

technique[a]	gradient (pH units)	aminopeptidase yield (%)	aminopeptidase purification (fold)	collagenase yield (%)	collagenase purification (fold)
chrom[b]	7.1-4.0	0	0	0	0
chrom[b]	7.1-3.0	32	1.8	7	0
rotofor[c]	3.0-10.0	21	1.4	18	0
rotofor[c]	3.2-3.9	14	0	10	0
rotofor[c]	3.9-5.6	10	0	5	0

[a] abbreviations: chrom, 5 mL chromatofocusing column from Pharmacia; rotofor cell from Bio-Rad for preparative isoelectric focusing.

[b] Chromatofocusing was performed by pre-equilibrating the column with 20 mM Bis-tris pH 7.1 and eluting with a 1 in 10 dilution of polybuffer 74 brought to a pH of either 4.0 or 3.0 with 2 M iminodiacetic acid. 0.5 mL of extracellular filtrate concentrate was loaded on the column for each run.

[c] The rotofor cell was run as per manufacturers instructions using one of ampholytes (3/10), bio-lytes (3.2/3.9) or bio-lytes (3.9/5.6) to create the gradient. 2.0 mL of extracellular filtrate concentrate was loaded into the rotofor cell for each run.

Table A2-3. Hydrophobic interaction chromatography column screening[a]

column[b]	material loaded	capacity (mL)	aminopeptidase yield (%)	aminopeptidase purification (fold)	collagenase yield (%)[d]	collagenase purification (fold)[d]
HP	ECF	amino-142 coll-47	100	28.5	54.2, 26.8	4.1, 4.2
LO	ECF	coll-32	100	73.0	73.0	46.9
HI	ECF	amino-148 coll -51	100	173.0	18.4, 41.1	43.2, 87.0
butyl	ECF		91.3	24.9	32.0, 42.2	8.2, 16.7
octyl	ECF		100	64.2	26.7, 11.0	5.2, 25.9
HP	HP peak 1				50.0	27.2
HP	HP peak 2				46.8 15.0	29.2 30.1
HI	HI peak 1					
HI	HI peak 2				18.2	39.9
HI[b]	ECF		100	140.0		
HI[b]	Q Seph amino pool		87.1	7.9		
LO	ECF				73.0	46.9
LO	Q Seph coll pool				42.3	3.8

[a] abbreviations: HP, phenyl sepharose high performance column; LO, low substitution phenyl sepharose column; HI, high substitution phenyl sepharose column; butyl, butyl sepharose column; octyl, octyl sepharose column; ECF, extracellular filtrate concentrate; HP or HI peak 1 or 2, one of the peaks of collagenase activity from the HP or HI columns re-loaded onto the same column; Q Seph, Q Sepharose Fast Flow anion exchange chromatography column pool; amino, aminopeptidase; coll, collagenase.

[b] All columns were 1 mL total volume and were run in 50 mM sodium phosphate, pH 7.0 with gradients from 1.0 - 0.0 M ammonium sulfate except for those indicated which were run from 0.5 - 0.0 M ammonium sulfate.

[c] Loading capacity was determined for the 1 mL columns by loading excess ECF onto the column and monitoring the material flowing through the column for activity. Volumes reported are the volume at which activity was detected flowing through the column.

[d] Two values are reported for the collagenase yield and purification in 4 of the 5 columns assayed because activity was detected in two distinct peaks.

Table A2-4. Reverse-phase chromatography column screening[a]

column	material loaded	buffer for elution	notes
C8	ECF ± DCI	TCA/acetonitrile	-no peak of collagenase activity detected
C8	Q Seph coll ± DCI	TCA/acetonitirile	-several peaks shifted after treatment with
C8	Phe LO coll ± DCI	TCA/acetonitrile	DCI
C8	ECF	TCA/acetonitrile (pH 2)	-no recovery of activity[b]
C8	ECF	phosphoric acid/ acetonitrile (pH 3)	-no recovery of activity[b]
C8	ECF	ammonium acetate/ acetonitrile (pH 6)	-no recovery of activity[b]

[a] abbreviations: C8, reversed phase column with an 8-carbon chain attached; ECF, extracellular filtrate concentrate; DCI, 3,4-dichloroisocoumarin; Q Seph coll, collagenase activity pool from the Q Sepharose Fast Flow anion exchange chromatography column; Phe LO coll, collagenase activity pool from the phenyl low substitution hydrophobic interaction chromatography column.

[b] No aminopeptidase or collagenase activity was recovered from any column fractions despite the fact that the buffers alone allowed retention of up to 8 % of activity and that was increased to up to 28 % by removal of the buffers by or dialysis.

Table A2-5. Methods for concentration of aminopeptidase and collagenase activities*

procedure	aminopeptidase yield (%)		collagenase yield (%)	
lyophilization	7.3		1.4	
centricon microconcentration	96.0		87.0	
polyethylene glycol concentration	59.9		25.6	
ammonium sulfate precipitation	supernatant	pellet	supernatant	pellet
20 %	80.0	0.0	50.0	0.0
40 %	74.0	0.0	20.0	0.0
60 %	0.0	62.0	7.0	42.0
80 %	0.0	58.0	3.0	45.0

* Experiments were performed concentrating 2 mL of extracellular filtrate concentrate 10-fold and assaying for aminopeptidase and collagenase activity before and after the procedure.

Table A2-6. Size exclusion chromatography column screening[a]

column	buffer	material loaded	aminopep yield (%)	aminopep purification (fold)	collagenase yield (%)	collagenase purification (fold)
G-100	PBS pH 7.4 .15 M NaCl	ECF	54.6	25.6	45.6	18.0
Superdex 200	PBS pH 7.4 .15 M NaCl	ECF	80.3	26.3	97.2	22.4
Superdex 200	PBS pH 7.4 .15 M NaCl	amino	62.7	2.6		
Superdex 200	PBS pH 7.4 .15 M NaCl	coll			91.3	10.1
Superdex 200	PBS pH 7.4 .15 M NaCl 0.1 % SDS	ECF	66.2	20.1		
Superdex 200	PBS pH 7.4 .15 M NaCl 0.1 % SDS	amino	58.8[b]	1.8[b]		

[a] abbreviations: G-100, Sephadex G-100 size exclusion chromatography column (30 mL); Superdex 200, Superdex 200 size exclusion chromatography column (30 mL); aminopep, aminopeptidase; ECF, extracellular filtrate concentrate; amino, aminopeptidase activity partially purified by AEC and HIC; coll, collagenase activity partially purified by AEC and HIC.

[b] Samples were dialyzed against three exchanges of 1000 volumes of PBS pH 7.4, 1 mM $ZnCl_2$ for 8 hours each prior to assaying activity recovered.

Table A2-7. Renaturation of aminopeptidase and collagenase activities[a]

denaturation conditions	dialysis buffer	aminopeptidase recovery (%)	collagenase recovery (%)
6 M GdnHCl[b]	PBS pH 7.4	38.2	1.5
0.1 % SDS[b]	PBS pH 7.4	31.0	0
8 M urea[b]	PBS pH 7.4	48.4	2.3
SDS-PAGE & electroelution[c]	PBS pH 7.4, 1mM $ZnCl_2$	97.1	0
SDS-PAGE & electroelution[c]	PBS pH 7.4, 1mM $CaCl_2$	28.4	0
SDS-PAGE & electroelution[c]	PBS pH 7.4, 1mM $MgCl_2$	0	0
SDS-PAGE & electroelution[c]	PBS pH 7.4, 1mM $MnCl_2$	0	0
SDS-PAGE & electroelution[c]	PBS pH 7.4, 1mM $NiCl_2$	0	0
SDS-PAGE & electroelution[c]	PBS pH 7.4, 1mM $CoCl_2$	0	0

[a] abbreviations: GdnHCl, guanidinium hydrochloride; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

[b] 1 mL of extracellular filtrate concentrate was denatured by adding the chemical listed to the concentration indicated and renatured by dialysis with 3 exchanges of 1000 volumes buffer for 8 hours each was performed.

[c] SDS-PAGE and electroelution were performed as described in Materials and Methods. Renaturation was performed by dialysis in the presence of the divalent metal cations indicated.

Table A2-8. Immobilized metal affinity chromatography screening for ligands and elution conditions for purification[a]

ligand	elution	aminopeptidase yield (%)	aminopeptidase purification (fold)
Mg^{2+}	batch - 1M NaCl	22.0	
Ca^{2+}	batch - 1M NaCl	26.1	
Zn^{2+}	batch - 1M NaCl	32.5	
Mn^{2+}	batch - 1M NaCl	13.6	
Ni^{2+}	batch - 1M NaCl	21.4	
Co^{2+}	batch - 1M NaCl	12.7	
Zn^{2+}	0.0-1.0 M NaCl	28.9	2.8
Zn^{2+}	1-10 mM EDTA	0.0	0
Zn^{2+}	pH 8.0-6.5-4.0	24.2	1.1

[a] Recovery of collagenase activity from batch experiments is less than 5 % of the activity loaded.

Table A2-9. Purification of collagenase by affinity chromatography matrices[a,b]

column matrix	material loaded	elution conditions	collagenase yield (%)	collagenase purification (fold)
PAB-Seph	ECF	pH 8.0-6.5-4.0	6.5	6.2
PAB-Seph	ECF	0-100 mM arg	13.1	14.2
PAB-Seph	ECF	0-1 M NaCl	13.4	14.4
PAB-Seph	Superdex 200 coll	0-1 M NaCl	10.8	1.1
arg-Seph	ECF	pH 8.0-6.5-4.0	6.4	4.4
arg-Seph	ECF	0-100 mM arg	10.8	11.8
arg-Seph	ECF	0-1 M NaCl	12.7	13.6
arg-Seph	Superdex 200 coll	0-1M NaCl	9.7	1.0

[a] abbreviations: PAB-Seph, para-amino-benzamidine sepharose affinity matrix from Pharmacia Biotech; arg-Seph, arginine-Sepharose affinity matrix from Pharmacia Biotech; ECF, extracellular filtrate concentrate; Superdex 200 coll, collagenase activity that has been partially purified by AEC, HIC and SEC.

[b] Collagenase activity did not bind to gelatin-sepharose or heparin-sepharose, 2 other affinity matrices tested.

Table A2-10. Gel electrophoresis systems screened for use in purification of the *M. tuberculosis* aminopeptidase and collagenase activities

technique	comments
multilocus enzyme electrophoresis	detect leucine aminopeptidase activity limitation: load less than 10 microliters
non-denaturing isoelectric focusing	detect aminopeptidase and collagenase using the synthetic substrates AAF-AMC and N-succinyl-GPLGP-AMC limitation: load less than 10 microliters
gelatin SDS-PAGE zymography	detect activity of gelatinase in extracellular filtrate concentrate limitation: the gelatinase activity does not co-elute with the collagenase activity partially purified therefore is due to a unique enzyme
substrate SDS-PAGE	unable to detect activity against the aminopeptidase or collagenase synthetic substrates
non-denaturing PAGE	unable to detect activity against the aminopeptidase or collagenase synthetic substrates
preparative SDS-PAGE	28.4 % of aminopeptidase activity was detected after dialysis of fractions in PBS pH 7.4 + 1 mM $ZnCl_2$ limitation: activity loaded is diluted approximately 100-fold

Appendix 3. Recipes for buffers, solutions and media used in the purification and characterization of the *M. tuberculosis* aminopeptidase and collagenase

50 mM ammonium acetate pH 4.0-7.5	3.85 g ammonium acetate 900 mL milliQ H_2O pH as required volume to 1 L with milliQ H_2O autoclave[a]
2 M ammonium sulfate	26.42 g ammonium sulfate volume to 100 mL with milliQ H_2O filter sterilize and degas[b]
basic fuschin	4.80 g basic fuschin volume to 100 mL milliQ H_2O
20 mM bis-tris pH 6.5	4.18 g bis-tris 900 mL milliQ H_2O pH to 6.5 volume to 1 L with milliQ H_2O filter sterilize and degas[b]
20 mM bis-tris pH 6.5, 1 M NaCl	4.18 g bis-tris 58.44 g sodium chloride 900 mL milliQ H_2O pH to 6.5 volume to 1 L with milliQ H_2O filter sterilize and degas[b]
25 mM bis-tris pH 7.1	5.23 g bis-tris 900 mL milliQ H_2O pH to 7.1 with 2M iminodiacetic acid volume to 1 L with milliQ H_2O filter sterilize and degas[b]
10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS), 10 % methanol pH 11.0	2.213 g CAPS 800 mL milliQ H_2O 100 mL methanol pH to 11.0 volume to 1 L with milliQ H_2O

carbonate buffer	8.40 g sodium carbonate (monobasic) 0.20 g magnesium chloride 900 mL milliQ H_2O pH to 9.8 volume to 1 L autoclave[a]
0.5 M ethylene diamine tetraacetic acid (EDTA) pH 8.0	186.1 g EDTA (disodium salt) 900 mL milliQ H_2O pH to 8.0 by dissolving sodium hydroxide pellets volume to 1 L with milliQ H_2O filter sterilize[b]
6 x Laemmli's digestion mix (LDM)	7.0 mL 0.5 M tris-HCl pH 8.0 3.0 mL glycerol 1.0 g sodium dodecyl sulfate 0.93 g dithiothreitol 1.2 mg bromophenol blue
Lowenstein-Jensen medium	4.0 g potassium phosphate (dibasic) 0.4 g magnesium sulfate 1.0 g magnesium citrate 6.0 g asparagine 20.0 mL glycerol volume to 1 L with distilled H_2O

Clean fresh eggs and break them into a sterile graduated cylinder. Take 1600 mL of egg fluid, mix in a screw-capped jar or polypropylene container with glass beads to break the yolks, filter through gauze and add to 1 L salt mixture. Add 50 mL of 1 % aqueous malachite green and dispense.

lysis buffer	13.4 g sodium phosphate (monobasic) 20 µL Tween 80 pH to 7.0 volume to 100 mL with milliQ H_2O autoclave[a]
phosphate-buffered saline (PBS) pH 7.4	8.00 g sodium chloride 0.20 g potassium chloride 1.44 g sodium phosphate (monobasic) 0.24 g potassium phosphate (dibasic) volume to 1 L with milliQ H_2O autoclave[a]

polybuffer 74 pH 3.0	10 mL polybuffer 70 mL milliQ H_2O pH to 3.0 with 2 M iminodiacetic acid
Ponceau S stain	0.2 g Ponceau S 3 g trichloroacetic acid volume to 100 mL with milliQ H_2O
1.0 mM sodium dodecyl sulfate	28.8 mg volume to 100 mL with milliQ H_2O
SDS-polyacrylamide gel running buffer (10 x)	30.2 g Tris base 144.0 g glycine 10.0 g SDS volume to 1 L with milliQ H_2O
12.5 % SDS-polyacrylamide separating gel mix	4.2 mL 30:0.8 acrylamide:bisacrylamide 1.3 mL 3.0 M tris pH 6.8 0.1 mL 10 % SDS 0.5 mL 1.5 % ammonium persulfate 4.0 mL H_2O 5.0 µL TEMED
5 % SDS-polyacrylamide stacking gel mix	0.63 mL 30:0.8 acrylamide:bisacrylamide 1.25 mL 0.5 M tris pH 6.8 0.05 mL 10 % SDS 0.25 mL 1.5 % ammonium persulfate 2.83 mL H_2O 4.0 µL TEMED
50 mM sodium phosphate pH 7.0	6.9 g sodium phosphate (monobasic) 900 mL milliQ H_2O pH to 7.0 volume to 1 L with milliQ H_2O filter sterilize and degas[b]
50 mM sodium phosphate pH 7.0, 0.15 M sodium chloride	6.9 g sodium phosphate (monobasic) 8.77 g sodium chloride 900 mL milliQ H_2O pH to 7.0 volume to 1 L with milliQ H_2O filter sterilize and degas[b]

50 mM sodium phosphate pH 7.0, 0.5 M ammonium sulfate	6.9 g sodium phosphate (monobasic) 66.1 g ammonium sulfate 900 mL milliQ H_2O pH to 7.0 volume to 1 L with milliQ H_2O filter sterilize and degas[b]
50 mM sodium phosphate pH 7.0, 1 M ammonium sulfate	6.9 g sodium phosphate (monobasic) 132.1 g ammonium sulfate 900 mL milliQ H_2O pH to 7.0 volume to 1 L with milliQ H_2O filter sterilize and degas[b]
Sauton's media	4.0 g L-asparagine 2.0 g citric acid 0.5 g potassium phosphate (monobasic) 0.5 g magnesium sulfate 0.25 g triton WR1339 0.05 g ferric ammonium citrate 40 mL glycerol volume to 1 L with milliQ H_2O pH to 7.1 mix, dispense and autoclave[a]
Towbin buffer	24.2 g tris base 7-9 (Sigma, St. Louis, MO, USA) 115.2 g glycine 1600 mL methanol volume to 8 L with milliQ H_2O check pH, which should be 8.3 autoclave[a]
50 mM Tris base pH 7.0-10.0	6.06 g tris base 900 mL milliQ H_2O pH as required volume to 1 L with milliQ H_2O autoclave[a]

[a] Autoclave at 15 pounds per square inch and 121°C for 20 min.

[b] Filter sterilize through a 0.45 micron filter under house vacuum and degas at 20 pounds per square inch for 30 min using a vacuum pump